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<p>(54) Title: DIACYLGLYCEROL ACYLTRANSFERASE GENE FROM PLANTS</p> <pre>     graph TD       G3P[Glycerol 3 Phosphate G3P] -- "Acyl-CoA, GPAT" --&gt; LPA[Lysophosphatidic Acid LPA]       LPA -- "Acyl-CoA, LPAT" --&gt; PA[Phosphatidic Acid PA]       PA -- "Acyl-CoA, PAPase" --&gt; DAG[Diacylglycerol DAG]       DAG -- "Acyl-CoA, DGAT" --&gt; TAG[Triglyceride TAG]   </pre>			
<p>(57) Abstract</p> <p>The present invention relates to the isolation, purification, characterization and use of the plant diacylglycerol acyltransferase (DGAT) gene and genetic products. For example, the invention provides DGAT cDNA [SEQ ID NO:1] (pDGATcDNA; ATCC No PTA-989) and a plant diacylglycerol acyltransferase gene [SEQ ID NO:3] (pDGATgene; ATCC No PTA-988) from the Brassicaceae (specifically <i>Arabidopsis thaliana</i>). The invention includes isolated and purified DGAT DNA, preferably of the stated sequences and homologues, and relates to methods of regulating seed oil content, the ratio of diacylglycerol/triacylglycerol proportions in the seed oil, fatty acid synthesis, seed oil acyl composition, seed size/weight and carbon flux into other seed components, using the gene, and to tissues and plants transformed with the gene. The invention also relates to transgenic plants, plant tissues and plant seeds having a genome containing an introduced DNA sequence of the invention, and a method of producing such plants and plant seeds. The invention also relates to [SEQ ID NO:1] containing an 81 bp insertion [SEQ ID NO:23], and uses thereof to modify oil content, acyl composition of triacylglycerols, seed size or carbon flux into other seed components.</p>			

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DIACYLGLYCEROL ACYLTRANSFERASE GENE FROM PLANTSTECHNICAL FIELD

This invention relates to plant genes useful for the genetic manipulation of plant characteristics. More specifically, the invention relates to the identification, isolation and introduction of diacylglycerol acyltransferase (DGAT) genes useful, for example, for altering the seed oil content, the ratio of diacylglycerol / triacylglycerol proportions in the seed oil, fatty acid synthesis, seed oil acyl composition, seed size/weight and carbon flux into other seed components, in commercial or crop plants.

10 BACKGROUND ART

Plant seed oils are major sources of essential polyunsaturated fatty acids for human diets and renewable feedstocks for chemical industries. The enzymes of the fatty acid synthase complex in the plastids of developing seeds are responsible for the biosynthesis of fatty acids that are channeled into the cytosolic acyl-CoA pool to sustain triacylglycerol accumulation. Triacylglycerol (TAG) biosynthesis is located in the endoplasmic reticulum with glycerol-3-phosphate and fatty acyl-CoAs as the primary substrates. There are three acyltransferases involved in the plant storage lipid bioassembly, namely the glycerol-3-phosphate acyltransferase (GPAT, EC 2.3.1.15), the lyso-phosphatidic acid acyltransferase (LPAT, EC 2.3.1.51) and the diacylglycerol acyltransferase (DGAT, EC 2.3.1.20). These three acyltransferases catalyze the stepwise acylation of the glycerol backbone with the final step being the acylation of *sn*-1, 2-diacylglycerol (DAG) by DGAT into the formation of TAGs, a biochemical process generally known as the Kennedy pathway (Stymne and Stobart, 1987).

25 Among the three ER-based fatty acyl-CoA acyltransferases, only LPAT gene(s) have been cloned from plants (Knutzon *et al.*, 1995, Lassner *et al.*, 1995). Like several other enzymes involved in storage lipid biosynthesis, acyltransferases are intrinsic ER proteins and are extremely difficult to purify. The research on plant DGAT has been largely limited to studies of activity profiles by using the particulate fractions generated by differential centrifugation of seed or microspore-derived embryo homogenates (Weselake *et al.*, 1993). Although partial purification of

DGAT from cotyledons of germinating soybean seeds was reported (Kwanyuan and Wilson, 1986), detailed molecular characterization of this enzyme is lacking.

Accordingly, while the Kennedy pathway is known and shows the steps in the biosynthesis of TAGs in plants, there has not been any identification and use of

- 5 a genetic element that can be used reliably in plants to modify TAG synthesis and composition in a way that may be exploited commercially.

#### DISCLOSURE OF THE INVENTION

An object of the invention is to identify, isolate and clone a genetic element that may be used to modify the natural formation of triacylglycerols in plants in order

- 10 to increase the yield of commercial plant oils, or to modify their composition to achieve specific commercial improvements of plants and plant products.

Another object of the invention is to identify, isolate and characterize diacylglycerol acyltransferase (DGAT) gene and cDNA sequences from *Arabidopsis* and to utilize these sequences in the genetic manipulation of plants.

- 15 Another object of the invention is to provide a vector containing the full-length DGAT cDNA sequence from *Arabidopsis* in a sense orientation under the control of a seed-specific promoter (e.g. napin; See Josefsson et al., 1987; Radke et al., 1988; Voelker et al., 1992), for re-introducing into *Arabidopsis* or for introducing into other plants.

- 20 Another object of the invention is to provide a vector containing a genomic fragment from *Arabidopsis* consisting of the full-length DGAT gene under the control of its own 5' upstream regulatory sequences, for re-introducing into *Arabidopsis* or for introducing into other plants.

- Another object of the invention is to provide a method to construct a vector  
25 containing the full-length DGAT sequence or a significant portion of the DGAT sequence from *Arabidopsis*, in an antisense orientation under control of either a constitutive or a seed-specific promoter, for re-introducing into *Arabidopsis* or for introducing into other plants.

Another object of the invention is to provide a method of modifying *Arabidopsis* and other plants to change their seed oil content.

Another object of the invention is to provide a method of modifying *Arabidopsis* and other plants to change the acyl composition of their seed oil.

5 Another object of the invention is to provide a method of modifying *Arabidopsis* and other plants to change their average seed weight or size.

According to one aspect of the present invention, there is provided a vector containing isolated and purified deoxyribonucleic acid (cDNA) of SEQ ID NO:1 (pDGATcDNA; ATCC No PTA-989), for introduction of the cDNA in a sense orientation into a plant cell.

As another aspect of the present invention, there is provided a vector containing isolated and purified genomic deoxyribonucleic acid (genomic DNA) of SEQ ID NO:3 (pDGATgene; ATCC No PTA-988), for introduction of the gene in a sense orientation into a plant cell.

15 According to yet another object of the invention, there is provided a method for preparing a vector containing SEQ ID NO:1 or a part thereof, for introduction of the gene or partial gene in an antisense orientation, into a plant cell.

According to yet another object of the invention, there is provided seed of *Arabidopsis thaliana* ecotype Columbia mutant AS11 (ATCC No. PTA-1013) and 20 characterization of its lipid phenotype (Katavic et al., 1995; Zou et al. 1999). The AS11 mutant seed line has an insertion mutation at the TAG1 locus on chromosome II, and produces plants exhibiting reduced DGAT activity (Figure 4) and an reduced TAG/DAG ratio during seed development (Table 1), resulting in an altered seed fatty acyl composition (Figure 2), reduced oil content (Table 1), and 25 increased seed oil diacylglycerol content during development (Figure 3) and at maturity (lower TAG/DAG ratio cf Table 1). The cDNA sequence of the AS11 DGAT is shown in SEQ ID NO:23, the genomic DNA sequence is shown in SEQ ID NO:24 and the translated protein sequence of the AS11 DGAT is shown in SEQ ID NO:25.

The invention also relates to transgenic plants and plant seeds having a genome containing an introduced DNA sequence of SEQ ID NO:1 or SEQ ID NO:3, and a method of producing such plants and plant seeds.

- The invention also relates to SEQ ID NO:1 or SEQ ID NO:3, or a part of
- 5 SEQ ID NO:1 or SEQ ID NO:3, or SEQ ID NO:1 containing an 81 bp insertion [SEQ ID NO: 23] or SEQ ID NO:3 containing an 147 bp insertion [SEQ ID NO:24] such that the deduced amino acid sequence of the encoded protein contains the repeated sequence SHAGLFNLCVVVLIAVNSRLIIENLMK [SEQ ID NO:25] where the spacing and identity of the underlined amino acids are identical or are replaced
  - 10 by conserved substitutions, characterized in that said sequence has been introduced in sense or antisense orientation, and a method of producing such plants and plant seeds.

- As will be appreciated by persons skilled in the art, the invention also relates to substantially homologous DNA sequences from plants encoding proteins with
- 15 deduced amino acid sequences of 25% or greater identity, and 40% or greater similarity, isolated and/or characterized and/or designed by known methods using the sequence information of SEQ ID NO:1 or SEQ ID NO:3 or or SEQ ID NO:1 containing an 81 bp insertion [SEQ ID NO:23] such that the deduced amino acid sequence of the encoded protein contains the repeated sequence
  - 20 SHAGLFNLCVVVLIVNSRLIIENLMK (SEQ ID NO:25) where the spacing and identity of the underlined amino acids are identical or are replaced by conserved substitutions, and to parts of reduced length that are still able to function as inhibitors of gene expression by use in an anti-sense, co-suppression (Transwitch; Jorgensen and Napoli 1994) or other gene silencing technologies. It will be
  - 25 appreciated by persons skilled in the art that small changes in the identities of nucleotides in a specific gene sequence may result in reduced or enhanced effectiveness of the genes and that, in some applications (e.g. anti-sense or co-suppression), partial sequences often work as effectively as full length versions. The ways in which the gene sequence can be varied or shortened are well known to
  - 30 persons skilled in the art, as are ways of testing the effectiveness of the altered genes. All such variations of the genes are therefore claimed as part of the present invention.

Other preferred degrees of identity to the indicated sequences are at least 30%, 40%, 50%, 60%, 70%, 80%, 90% and 95%; and other preferred degrees of similarity are at least 50%, 60%, 70%, 80%, 90% and 95%. The inventors have used a computer program known as MegAlign®, DNASTAR® or DNASTAR Inc.,

- 5 1228 South Park Street, Madison, WI 53715, USA, for assessing homology. This program is based on the Clustal V algorithm (Higgins and Sharp (1998): A package for performing multiple sequence alignment on a microcomputer; GENE 73:237-244). For each gap introduced in the alignment, the program deducts a penalty from the score. A higher gap penalty suppresses gapping; a lower value promotes  
10 it. The program also assesses penalties based on the length of the gap. The more residues the gap spans, the greater the penalty. The program deducts these penalties from the overall score of the alignment.

Stated more generally, the present invention relates to the isolation, purification and characterization of a diacylglycerol acyltransferase (DGAT) gene  
15 from the Brassicaceae (specifically *Arabidopsis thaliana*) and demonstrates its utility in regulating fatty acid synthesis, seed oil content, diacylglycerol/triacylglycerol ratios and seed size/weight. Until now, no concrete data is available on the gene structure of plant DGATs, or their utility in altering oil content or composition through genetic manipulation.

- 20 When considering altered oil contents or compositions, results from averages of statistically-significant numbers of plants or seeds according to the invention are best compared with results from averages of statistically-significant numbers of untransformed (control) plants or seeds of the same genotype grown under identical conditions at the same time. This allows for the variability of  
25 individual plants of the same genotype, particularly when such plants are grown under different conditions. The actual number of plants or seeds used to form the required average may vary, but should be enough to provide a generally constant average whenever such number is selected. Generally, the number should be at least 10, and is more preferably at least 20, 30, 50 or 100.  
30 The DGAT gene was cloned, characterized and authenticated from *Arabidopsis* by: (1) selection and characterization of plant ESTs sharing some homology to mammalian acyl-CoA: cholesterol acyltransferases; (2) the functional

expression of a full-length cDNA in yeast; (3) the characterization and isolation of the DGAT (*TAG1*) gene from *Arabidopsis* mutant AS11 containing an insertion mutation in the DGAT gene and a seed oil phenotype which consists of an altered DAG/TAG ratio, and an altered oil content and acyl composition; (4)

- 5 complementation of the AS11 mutant by insertion of the DGAT cDNA sequence to restore a wild-type fatty acid composition; (5) the over-expression of the DGAT cDNA in wild-type *A. thaliana* transgenic plants which produce seeds with an increased oil content, increased average seed weight and altered seed oil acyl composition.

10 The *A. thaliana* DGAT structure is significantly homologous (over 40% identical over a region of more than 400 amino acids) to its mammalian counterparts, and is highly homologous to subsequently reported putative *B. napus* DGATs at both the nucleotide (92%) and the deduced amino acid (90%) levels (Nikyiforuk et al, 1999; GenBank/EMBL Accession No AF155224; AF164434).

15 The DGAT of the current invention is useful in manipulating DGAT activity, and triacylglycerol bioassembly in plants. For example, by transforming plants with a construct containing the DGAT gene in a sense orientation, under the control of a tissue-specific promoter (e.g. seed-specific promoter napin), the expression of DGAT and accumulation of seed oil can be enhanced or the acyl composition of the 20 seed oil altered. Yet another example would be to express the DGAT cDNA under the control of a constitutive promoter (e.g. 35S (Datla et al., 1993)) to increase the TAG content of vegetative tissues (leaves, roots, stems). This may have particular advantages for altering the starch/oil ratio in root crops.

25 Alternatively, DGAT expression can be silenced to some degree by anti-sense or co-suppression (Transswitch) phenomena (De Lange et al., 1995; Mol et al., 1990; Jorgensen and Napoli, 1994; Kinney, 1995; Vaucheret et al, 1998; Taylor, 1998). For example, silencing DGAT in a seed specific manner may result in a reduction in TAG accumulation. This could have applications in reducing the oil content in seed barley to enhance stability during storage. As a second example, 30 seed-specific silencing may lead to a relatively high accumulation of DAG or an increase in the DAG/TAG ratio in the developing or mature seed. As yet another example, the expression of the mutated DGAT gene which results in a 27 amino

- acid repeat insertion in the mutant DGAT protein (See Figure 5 a) can be used to alter the DAG/TAG ratio in developing and mature seed. Such manipulations can lead to edible seed oils produced naturally in the plant, containing enhanced relative levels of DAG /reduced levels of TAG (See Figure 3; Table 1) to act as all-natural emulsifiers in the food and confections industries, or to enhance the nutritional/health profile of vegetable oils as functional foods (e.g. as cooking oils, stir fry oils, in salad dressings, margarines etc.) by inhibiting neutral fat deposition in humans. Processed oils produced from canola and soybean which contain increased proportions of diacylglycerol have been cited by the Kao Corporation of Japan (e.g. Econa Cooking Oil; Kao Corporation KI, 1-14-10 Nihonbashi-Kayabacho, Chuoku, Tokyo 103 Japan; e-mail: 210064@kastanet.kao.co.jp) as a product making it difficult for blood neutral fat to increase after a meal, and for fat to cling to the body, thereby assisting individuals who are overweight or who suffer from high neutral fat levels. As a third example, silencing or reducing the activity of DGAT in a seed specific manner (as observed in mutant AS11; e.g. by over-expressing SEQ ID NO:23 or silencing expression of SEQ ID NO:1 or SEQ ID NO:3), and combining this trait with the capacity to produce polyhydroxyalkanoates (PHAs; e.g. polyhydroxybutyrate) in seeds (Poirier et al., 1992; 1995) will allow an increased flow of unesterified fatty acids towards  $\beta$ -oxidation (Poirier et al., 1999). By recycling or diverting the unesterified fatty acids into  $\beta$  oxidation, the resulting acetyl-CoA moieties will lead to a significant increase in polyhydroxyalkanoates (PHAs) or a change in PHA composition (Poirier et al., 1999). Transgenic plants producing PHAs in seeds have potential for utility as biodegradeable thermoplastics. However, up to now, the levels of PHAs produced have been relatively small (Poirier et al, 1992; 1995). The utility of transgenically reducing the DGAT activity to significantly enhance PHA production (e.g. 10-fold increase) in PHA-producing seeds is now possible, due to the current DGAT invention.

- Some of the manipulations and deliverables which are possible using the DGAT gene or a part therof, include, but are not limited to, the following: seeds with increased or decreased oil content; seeds containing oils with an enhanced diacylglycerol content, seed oils with an altered acyl composition; plants producing larger or heavier seeds; plants exhibiting an enhanced or altered capacity to accumulate storage compounds in other storage organs (e.g. tubers, roots).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram illustrating the Kennedy pathway for the bioassembly of triacylglycerols in plants, and shows the critical role played by DGAT as the final step of the Kennedy pathway.

5       Figure 2 is a graph showing the comparison of the fatty acid composition of seed oil from wild-type (WT) and DGAT mutant AS11 lines of *Arabidopsis thaliana*. Proportions of fatty acids are reported as Mol % of the total fatty acid composition of the seed oil from each line. Error bars are  $\pm$  SE ( $n= 10$  plants of each line sampled, with 50 seeds per sample per analysis).

10      Figure 3 is a graph showing a comparison of the DAG content in developing seed of wild-type (WT) and DGAT mutant AS11 lines of *Arabidopsis thaliana*. More specifically, it is a comparison of the fatty acid content of DAG pool in wild-type green developing seeds compared to that of the AS11 mutant.

Figure 4 is a graph showing a comparison of the DGAT activity  
15 (pmol/min/mg protein) in developing seeds at the milky, early green, mid-green and brown-green stages of embryo development in wild-type (WT) and DGAT mutant AS11 lines of *Arabidopsis thaliana*. Developing seeds at each stage were selected and DGAT enzyme analyses conducted as described previously by Katavic et al., 1995).

20      Figure 5(a) shows the amino acid sequence alignment of the *Arabidopsis* DGAT (AtTAG1) [SEQ ID NO:2] with mammalian (mouse and putative human) DGATs. MDGAT, mouse DGAT [SEQ ID NO:4]; GenBank/EMBL Accession No. AF078752 (Cases et al., 1998); HARGP1, human ARGP1 protein [SEQ ID NO:5]; GenBank/EMBL Accession No. AF059202; Oelkers et al., 1998]. Dots indicate  
25 gaps. Identical amino acid residues are highlighted in black. Conserved residues are shaded. The 27-amino acid repeat found in *Arabidopsis thaliana* mutant AS11 and generated by the insertion mutation (81 bp) found in SEQ ID NO:23 (SHAGLFNLCVVV/LIAVNSRLIIENLMK) is overlined thus: \_\_\_\_\_. The putative diacylglycerol binding site is overlined thus: \_\_\_\_\_. The SnRK1 targeting site is  
30 overlined thus: \_\_\_\_\_ with an asterisk (\*) over the serine (S) residue as the targeted phosphorylation site.

Figure 5(b) shows the Kyte-Doolittle hydropathy plot of the DGAT protein.

Figure 6(a) shows the results of a Northern analysis of *TAG1* gene expression in *Arabidopsis thaliana*. Total RNA was extracted from roots (RT), leaves (LF), flowers (FL), young seedlings (YS), developing siliques (SL), and 5 germinating seeds (GS).

Figure 6(b) shows the results of Southern blot analysis of the *TAG1* gene in *Arabidopsis thaliana*. Genomic DNA was digested with restriction enzymes *Bgl*II (Lane 1), *Eco*RI (Lane 2), and *Hind*III (Lane 3). The *TAG1* DNA probe was  $^{32}\text{P}$  labeled by random priming.

10 Figure 7(a) is a diagrammatic representation of the *TAG1* gene structure. The boxes indicate the 16 exons (solid boxes for coding regions, open box for untranslated regions), and the lines represent the 15 introns. A, B and C denote the positions of the primers used for PCR amplifications of the segments from wild type (WT) and AS11. The specific primers A, B and C are described in Experimental  
15 Procedures: *Primer Strategy* (found later in this specification).

Fig 7(b) shows gel separation of the PCR products amplified from wild type (WT) and AS11. Lane 1, PCR product with primers A and B using WT genomic DNA as template. Lane 2, PCR product with primers A and B using AS11 genomic DNA as template. Lane 3, PCR product with primers C and B using WT genomic  
20 DNA as template. Lane 4, PCR product with primers C and B using AS11 genomic DNA as template. Lane 5, RT-PCR with primers A and B using RNA prepared from WT seedling RNA. Lane 6, RT-PCR with primers A and B using RNA prepared from AS11 seedling RNA.

Figure 8 is a graph showing microsomal DGAT activity in Yeast Strain YMN5  
25 Transformed with empty plasmid (pYES Con) and with the *A. thaliana* DGAT cDNA (pYES:DGAT). This illustrates the expression of the *TAG1* cDNA in yeast. Host cultures of strain YMN5 were transformed with pYES2 plasmid only (pYES2; without *TAG1* insert) or with pYES2 containing the *TAG1* cDNA insert (pYES2:TAG1). Following induction in the presence of galactose, transformants were lysed and  
30 assayed for DGAT activity as described in the Experimental Procedures. The results of two separate DGAT activity experiments are shown.

Figure 9 is a map of plasmid pSE129A which may be used as a vector. The vector contains the following salient features for plant transformation in the current invention: the seed-specific napin promoter and NOS terminator between which is a multiple cloning site.

5       Figure 10 is a graph showing the complementation of the AS11 DGAT mutation with the wild-type cDNA. Transformation of *Arabidopsis thaliana* mutant line AS11 with the DGAT cDNA [SEQ ID NO:1] under the control of a napin promoter, leads to a restoration of the wild-type (WT) fatty acid composition in the seed oil of the transformant lines 3-4, 9-1, 14-2 and 9-4. Fatty acid composition (wt 10 %) was determined on the seed oil extracted from 100-seed samples from *A. thaliana* non-transformed controls (WT), mutant line AS11, and T<sub>2</sub> seeds of napin:DGAT transgenic lines.

Figure 11 is a graph showing the seed oil content of non-transformed WT control, and pRD400 control (empty plasmid) and napin:DGAT T<sub>2</sub> transgenic 15 *Arabidopsis thaliana* seed lines. More particularly, the graph shows the transformation of wild type (WT) *Arabidopsis thaliana* with the DGAT cDNA [SEQ ID NO:1] under the control of a napin promoter, leads to a higher seed oil content in the DGAT transgenic lines. Oil content is expressed as µg total fatty acids (FAs) per 100 seeds from *A. thaliana* non-transformed controls (WT Con), and T<sub>2</sub> seeds of 20 pRD400 control (empty plasmid) transgenic, and napin:DGAT transgenic lines 1', 2', 9, 10 and 11. Standard error bars for the control lines are indicated; n=10.

Figure 12 is a graph showing the average 100-seed weight of non-transformed WT control, and pRD400 control (empty plasmid) and napin:DGAT T<sub>2</sub> transgenic *Arabidopsis thaliana* seed lines. More specifically, the graph shows the 25 over-expression of the DGAT cDNA under the control of a napin promoter, in wild-type (WT) *Arabidopsis thaliana* leads to a higher average seed weight. The average weight of 100-seed samples from *A. thaliana* non-transformed controls (WT Con), and T<sub>2</sub> seeds of pRD400 control (empty plasmid) transgenic, and napin:DGAT transgenic lines 1', 2', 9, 10 and 11 are reported as mg dry weight (DW).

30       Figure 13 is a graph showing the positive correlation between oil content (expressed as µg Total fatty acids (FAs) per 100 seeds) and average seed weight (expressed as average mg DW per 100-seed samples) from *A. thaliana* non-

transformed controls (WT Con ♦), and T2 seeds of pRD400 control (empty plasmid) transgenic (○) and napin:DGAT transgenic lines 1', 2', 9, 10 and 11 (■) are reported as mg dry weight (DW).

### BEST MODES FOR CARRYING OUT THE INVENTION

- 5        Fig. 1 is a diagram illustrating the Kennedy pathway for the biosynthesis of TAGs in plants. Of the various illustrated enzymes, DGAT is the only enzyme in the Kennedy pathway that is exclusively committed to TAG biosynthesis, and its key role is apparent from the scheme of Figure 1. *sn*-1,2-DAG, generated as a result of either the catalytic action of PA phosphatase (EC 3.1.3.4) or CPTase (EC 2.7.8.2),  
10 can be used in the biosynthesis of TAG.

For this reason, the inventors of the present invention decided to investigate DGAT to see if the corresponding gene in plants could be sequenced and cloned and used to modify the seed oil content and fatty acid composition of plants in a way that could be commercially useful.

- 15       The acyl-CoA dependent acylation of *sn*-1,2-DAG is catalyzed by DGAT (Stymne and Stobart, 1987). In developing and germinating seeds of oilseed plants, TAG accumulation and DGAT activity have been shown to associate with the endoplasmic reticulum (ER; high speed microsomal fraction) (Stobart et al., 1986; Cao and Huang, 1986; Stymne and Stobart, 1987; Frentzen, 1993; Settlage et al.,  
20 1995; Lacey and Hills, 1996). The biochemical properties of microsomal DGAT have been examined in a number of plant systems (Frentzen, 1993) including developing seeds (Bernerth and Frentzen, 1990; Vogel and Browse, 1996; Cao and Huang, 1987) and embryo cultures (Taylor et al., 1991; Weselake et al., 1991; Taylor et al., 1992; Little et al., 1994) of *B. napus* L. In general, studies with developing seeds  
25 indicate that DGAT activity increased rapidly during the active phase of oil accumulation and then decreases markedly as seed lipid content reaches a plateau (Tzen et al., 1993; Weselake et al., 1993).

- A number of studies with both mammalian (Mayorek et al., 1989; Tijburg et al., 1989) and plant (Ichihara et al., 1988; Perry and Harwood, 1993 a and 1993 b;  
30 Settlage et al. 1995) systems have suggested that DGAT may catalyze a rate-limiting reaction in TAG bioassembly. However, this hypothesis has not been

- rigorously tested, and has not been reduced to practice by transgenic expression of any DGAT gene in plant or animal systems, until now. Developing seeds of *B. napus* L., cv Shiralee, have been shown to produce significant levels of DAG during the active phase of oil accumulation suggesting that DGAT catalyzed
- 5 reaction may regulate the flow of carbon into TAG (Perry and Harwood, 1993 a and 1993 b). In addition, an ethyl methanesulfonate-induced (EMS-induced) mutant of *A. thaliana*, designated AS11, has been shown to have a reduced DGAT activity that correlated with both an increased DAG pool and decreased accumulation of TAG (Katavic et al. 1995). Given its possible rate-limiting role in TAG bioassembly,
- 10 the inventors of the present invention have identified DGAT as a potential target in the genetic modification of plant lipid biosynthesis.

Previously, the partial characterization of an EMS-induced *Arabidopsis thaliana* mutant, AS11, with altered fatty acid composition was reported (Katavic et al., 1995). In comparison to wild type plant seeds, AS11 seeds have reduced levels

15 of the very long chain fatty acid eicosenoic acid (20:1) and reduced oleic acid (18:1) and accumulate  $\alpha$ -linolenic acid (18:3) as the major fatty acid in triacylglycerols (Figure 2). The AS11 mutant has a consistently lower ratio of TAG/DAG in developing seeds, and it accumulates an elevated amount of seed DAG (Figure 3), the substrate of the diacylglycerol acyltransferase. Through a series of biochemical

20 analyses, it was shown that AS11 had reduced diacylglycerol acyltransferase activities throughout seed development (Figure 4). AS11 also had a reduced (by 25-30%) oil content phenotype, providing some evidence that DGAT may be controlling flux into TAG biosynthesis, as shown in Table 1 below. The AS11 did not have a wrinkled-seed phenotype as described in other low-seed-oil mutants (Focks

25 and Benning, 1998).

Table 1

Comparison of AS11 [Katavic et al., (1995) and wild-type *A. thaliana* seeds with respect to lipid profiles at mid-development, and the relative TAG, DAG and sterol ester contents in AS11 and WT seeds at maturity.

Seed Type	TAG/DAG ratio at mid- development <sup>a</sup>	TAG/DAG ratio at maturity <sup>a</sup>	Relative TAG content at maturity <sup>b</sup> <sup>c</sup> (nmol/mg DW)	Sterol Esters at maturity (% of Total Lipid Extract) <sup>d</sup>
WT	17	90	1.00 <sup>b</sup> <sup>c</sup> (255)	0.8
AS11	5	20	0.6 <sup>b</sup> <sup>c</sup> (174)	1.15

<sup>a</sup> Embryos staged and lipids measured as described in Katavic et al., (1995);

<sup>b</sup> Relative TAG content of 200-seed samples of AS11 and WT were measured by MASS-1H-NMR according to the method of Rutar (1989). The integration response for resonances attributable to liquid-like oil were summed and the value for AS11 seed is reported relative to the response for the WT control seed sample (the latter set at a value of 1.00); <sup>c</sup> TAG content (nmoles/mg DW) measured by transmethylation of a TLC-purified TAG fraction, followed by GC analysis of fatty acid methyl esters; <sup>d</sup> A total lipid extract was prepared as described by Taylor et al., (1991; 1992), and sterol esters isolated and characterized as described in the Experimental Procedures.

Genetic analysis indicated that the fatty acid phenotype is caused by a semidominant mutation in a nuclear gene, designated *TAG1*. The mutation was mapped to chromosome II, and was estimated to lie in the region approximately 17.5 + 3 cM from the *st1* locus and 8 + 2 cM from the *cp2* locus.

5 Because a DGAT gene has not heretofore been cloned from any plant, until now, it has not been possible to address the possibility of genetic modifications to alter carbon flux, increase fatty acid synthesis, oil content, oil acyl composition, or seed size, by modulating plant DGAT activity.

However, there are many examples of successful modifications to plant  
10 metabolism that have been achieved by genetic engineering to transfer new genes or to alter the expression of existing genes, in plants. It is now routinely possible to introduce genes into many plant species of agronomic significance to improve crop performance (e.g. seed oil or tuber starch content/composition; meal improvement;

herbicide, disease or insect resistance; heavy metal tolerance etc.) (MacKenzie and Jain, 1997; Budziszewski et al., 1996; Somerville, 1993; Kishore and Somerville, 1993).

- For example, increases in the proportions of some strategic fatty acids and
- 5 in the quantities of seed oil have been achieved by the introduction of various fatty acid biosynthesis and acyltransferase genes in oilseed crops. These include the following demonstrations: Expression of an anti-sense construct to the stearoyl-ACP  $\Delta 9$  desaturase in Brassicaceae led to an increase in the stearic acid content (Knutzon et al., 1992). Expression of a medium chain fatty acyl-ACP thioesterase
- 10 from California Bay, in Brassicaceae was demonstrated to increase the lauric acid (12:0) content (Voelker et al., 1992; 1996). Expression of a Jojoba  $\beta$  keto-acyl-CoA synthase in low erucic acid Brassicaceae led to an increase the level of erucic acid (22:1); the effect following expression in high erucic acid cultivars was negligible (Lassner et al., 1996). Increased proportions of oleic acid in *Brassica napus* and in
- 15 soybean have been achieved by silencing the microsomal FAD2 ( $\Delta 12$ ) desaturase (Hitz et al., 1995; Kinney, 1995 ; 1997). Transformation of *Arabidopsis thaliana* and rapeseed (*B. napus*) with a yeast sn-2 acyltransferase resulted in seed oils with increased proportions of 22:1 and other very long-chain fatty acids and significant increases in seed oil content (Zou et al., 1997).
- 20 Starch deposition has also been altered by genetic engineering. By expression of a mutant *E. coli* *glgC16* gene encoding an ADP glucose pyrophosphorylase in potato tubers, an increase in starch accumulation was achieved (Stark et al., 1992).

The inventors therefore considered the DGAT gene to hold great promise for

25 the desired modification of TAGs in plants.

The best modes for carrying out the invention will be apparent from the following description of the results of tests and experiments that have been carried out by the inventors.

The inventors chose to use the well-accepted model plant system

30 *Arabidopsis thaliana* for the cloning of DGAT, as a host system for genetic engineering to alter DGAT expression, and to study the effects of altering DGAT

expression on seed triacylglycerol bioassembly. This is because, over the past several years, *Arabidopsis thaliana*, a typical flowering plant, has gained increasing popularity as a model system for the study of plant biology. As a result of the ease with which this plant lends itself to work in both classical and molecular genetics,

- 5   *Arabidopsis* has come to be widely used as a model organism in plant molecular genetics, development, physiology and biochemistry (Meyerowitz and Chang, 1985; Meyerowitz, 1987; Goodman et al., 1995). This model dicotyledonous plant is also closely related to *Brassica* crop species and it is increasingly apparent that information concerning the genetic control of basic biological processes in
- 10   *Arabidopsis* will be transferable to other species (Lagercrantz et al., 1996).

Indeed, there are numerous examples wherein studies of the molecular biology and biochemistry of a particular metabolic pathway or developmental process and the possibility of genetically engineering a plant to bring about changes to said metabolic pathway or process, has first been tested in the model plant

- 15   *Arabidopsis*, and then shown to yield similar phenotypes in other plants, particularly crop plants.

- For example, the extra- plastidial membrane associated oleate (18:1) Δ12 (ω-6) desaturase gene, *FAD2*, was originally studied and eventually cloned from *Arabidopsis thaliana*, by identifying the lesion found in an *A. thaliana* mutant
- 20   defective in desaturating oleate to produce linoleate (18:2) on the phosphatidylcholine backbone. This resulted in a high oleic acid phenotype in the *A. thaliana* seed oil (Okuley et al., 1994). Genetic engineering of both soybean (*Glycine max.*) and canola *B. napus* to silence the indigenous *FAD2* gene(s) in a seed-specific manner by anti-sense or co-suppression approaches, resulted in
  - 25   similar high oleic acid seed oil phenotypes (Kinney, 1995; 1997).

- Transgenic expression of a yeast sn-2 acyltransferase (*SLC1-1*) gene to achieve enhanced seed oil and very long-chain fatty acid content was first performed in *Arabidopsis* and later shown to yield similar phenotypes in transgenic rapeseed (*B. napus*) experiments (Zou et al., 1997). *Arabidopsis thaliana* has
- 30   repeatedly shown itself to be a useful model system for metabolic engineering of metabolic pathways (e.g. lipid biosynthesis, photosynthesis) or processes (organogenesis, reproductive development etc.) common to all higher plants.

In the area of secondary metabolism/signal transduction, an anthocyanin pathway-specific transcriptional activator from the monocot maize designated as *R* (the *myc* transcription factor involved in activation of biosynthetic genes for anthocyanin production in the aleurone cells of maize kernels), was expressed in 5 the dicot *Arabidopsis*, causing augmented anthocyanin pigmentation in the inflorescences. Subsequent expression in another dicot, tobacco (*Nicotiana tabacum*), resulted in similar floral pigmentation changes (Lloyd et al., 1992). These experiments demonstrate that whole pathways common to all flowering plants can be co-ordinately controlled through the introduction of transcriptional regulators, and 10 that the mechanisms are common to diverse plant species.

In the context of the current invention, all plant seeds accumulate some triacylglycerol (oil) and this ubiquitous process is affected, at least in part, by the activity of a microsomal DGAT, as explained previously. Thus, many of the effects observed following genetic engineering to modulate DGAT expression in 15 *Arabidopsis* can be expected and predicted to result in similar phenotypes when carried out in all other plants. For example, after the present invention was made, information has become available that supports the findings of the present inventors by showing that *B. napus* has a highly homologous DGAT gene (Nikiforuk et al., 1999), and thus *B. napus* is a clear target for similar genetic modifications as those 20 shown for *A. thaliana*.

There are a number of ways by which genes and gene constructs can be introduced into plants, and a combination of plant transformation and tissue culture techniques have been successfully integrated into effective strategies for creating transgenic crop plants. These methods, which can be used in the present 25 invention, have been extensively reviewed elsewhere (Potrykus, 1991; Vasil, 1994; Walden and Wingender, 1995; Songstad et al., 1995), and are well known to persons skilled in the art. For example, one skilled in the art will certainly be aware that, in addition to *Agrobacterium*-mediated transformation of *Arabidopsis* by vacuum infiltration (Bechtold et al., 1993) or wound inoculation (Katavic et al., 30 1994), it is equally possible to transform other plant and crop species, using *Agrobacterium* Ti-plasmid-mediated transformation (e.g. hypocotyl (DeBlock et al., 1989) or cotyledonary petiole (Moloney et al., 1989) wound infection), particle bombardment/biolistic methods (Sanford et al., 1987; Nehra et al., 1994; Becker et

al., 1994) or polyethylene glycol-assisted protoplast transformation (Rhodes et al., 1988; Shimamoto et al., 1989) methods.

As will also be apparent to persons skilled in the art, and as extensively reviewed elsewhere (Meyer, 1995; Datla et al., 1997), it is possible to utilize plant promoters to direct any intended up- or down-regulation of transgene expression using constitutive promoters (e.g. those based on CaMV35S), or by using promoters which can target gene expression to particular cells, tissues (e.g. napin promoter for expression of transgenes in developing seed cotyledons), organs (e.g. roots), to a particular developmental stage, or in response to a particular external stimulus (e.g. heat shock).

Particularly preferred plants for modification according to the present invention include *Arabidopsis thaliana*, borage (*Borago* spp.), Canola, castor (*Ricinus communis*), cocoa bean (*Theobroma cacao*), corn (*Zea mays*), cotton (*Gossypium* spp.), *Crambe* spp., *Cuphea* spp., flax (*Linum* spp.), *Lesquerella* and 15 *Limnanthes* spp., Linola, nasturtium (*Tropaeolum* spp.), *Oenothera* spp., olive (*Olea* spp.), palm (*Elaeis* spp.), peanut (*Arachis* spp.), rapeseed, safflower (*Carthamus* spp.), soybean (*Glycine* and *Soja* spp.), sunflower (*Helianthus* spp.), tobacco (*Nicotiana* spp.), *Vernonia* spp., wheat (*Triticum* spp.), barley (*Hordeum* spp.), rice (*Oryza* spp.), oat (*Avena* spp.) sorghum (*Sorghum* spp.), rye (*Secale* spp.) or other 20 members of the Gramineae.

The present invention is particularly useful when used to modify the yield or composition of oilseed produced from oilseed crops. Oilseed crops are plant species that are capable of generating edible or industrially useful oils in commercially significant yields, and include many of the plant species listed above. 25 Such oilseed crops are well known to persons skilled in the art.

## RESULTS

### *Isolation of the TAG1 (DGAT) cDNA from Arabidopsis thaliana*

Since one of the most likely defects in AS11 mutant is at the DGAT itself (Table 1; Fig 4), the inventors attempted cloning strategies based on sequence 30 information of enzymes that share common substrates with DGAT. One of the

candidate enzymes that would serve this purpose is the acyl-CoA: cholesterol acyltransferase (ACAT, EC 2.3.1.26) (Chang *et al.*, 1997). Like DGAT, ACAT is an ER protein functioning as an O-acyltransferase by using acyl-CoA as the fatty acyl donor for the esterification of free cholesterol to generate sterol esters. Through a

- 5 BLAST database search, the inventors identified an *Arabidopsis thaliana* expressed sequence tag (EST) [accession no. AA042298; SEQ ID NO:6] with a deduced amino acid sequence showing 41 % identity to that of the yeast acyl-CoA: cholesterol acyltransferase (Yang *et al.*, 1996, Yu *et al.*, 1996), within the short sequence (104 amino acids) that was available for the EST.
- 10 The corresponding cDNA (E6B2T7) clone was obtained from the *Arabidopsis* Biological Resource Center, Columbus, Ohio. Upon complete sequencing, the 878 bp E6B2T7 clone was found to be a partial cDNA. However, the ORF prediction from this partial cDNA confirmed the initial EST search results in that the encoded product is structurally similar to ACAT, especially in the regions at
- 15 the C-terminus. The inventors were confident that the cDNA contained the 3' untranslated region through an ORF search, although the polyA tail was missing.

The inventors further used the partial cDNA sequence to search against *Arabidopsis thaliana* genomic sequence information. An *Arabidopsis* 'IGF' BAC clone 'F27F23' [accession no. AC003058] was identified to include a region that

20 matched the cDNA, and therefore it was concluded that this was the region encompassing the corresponding gene. Moreover, this BAC clone 'F27F23', is contained in the YAC clone, CIC06E08, which, according to the published map position ([http://weeds.mgh.harvard.edu/goodman/c2\\_b.html](http://weeds.mgh.harvard.edu/goodman/c2_b.html)), represents a region between centimorgan 35.9 and centimorgan 38.7 on chromosome II; this position is

25 similar to the estimated location for TAG1, and the lesion identified by the mutation in AS11 (Katavic *et al.*, 1995). In view of our previous results on the characterization of the AS11 mutant, the map position of this gene strongly suggested that it may encode a DGAT.

To clone a full-length cDNA, a series of oligonucleotide primers were

30 designed, based on the genomic sequences located at different positions 5' upstream of the region covering the partial cDNA. We used these primers in combination with a primer located at the 3' UTR of the partial cDNA (E6B2T7) to

perform PCR reactions with cDNA phagemid prepared from an *Arabidopsis thaliana* (ecotype Columbia) silique-specific cDNA library (Giraudat et al., 1992) as a template. The longest cDNA amplified was 1904 bp, which we subsequently designated as TAG1, and deposited into the Genbank under accession AJ238008

- 5 [SEQ ID NO:1]. We believe this cDNA represents a full-length clone because its size is in agreement with that of the transcript detected in the northern blot (see Figure 6 a). The longest open reading frame is flanked by a 134-nucleotide 5' untranslated region and a 203-nucleotide 3' untranslated region. There is an in-frame stop codon (TGA at position nt-43) which is followed by an in-frame ATG at  
10 position nt-139. It is thus inferred that the ATG at position nt-139 is the initiation codon.

The primary structure of TAG1 predicts a DGAT-related enzyme

- The predicted open reading frame of the TAG1 cDNA encodes for a polypeptide of 520 amino acids with a calculated molecular weight of 58993  
15 Daltons. With the BLAST search program (Altschul et al. 1990), it was found that the recently reported mouse diacylglycerol acyltransferase [accession no. AF078752] (Cases et al., 1998) is a protein which showed the highest sequence similarity to the deduced amino acid sequence of TAG1 (Figure 5a). TAG1 was also similar to a human acyl CoA: cholesterol acyltransferase-related enzyme  
20 [accession no. AF059202]. The human acyl CoA: cholesterol acyltransferase-related enzyme, also known as ARGP1, is most likely to be a DGAT with no significant ACAT activity, although the true nature of the enzyme awaits further confirmation (Oelkers et al., 1998). The similarity between TAG1 and the mammalian DGAT extends over a region of more than 400 amino acids with a  
25 sequence identity of about 41%. A putative diacylglycerol/phorbol ester-binding motif, HKW-X-X-RH-X-Y-X-P, a signature sequence observed to be unique to DGAT while absent in the ACATs (Oelkers et al., 1998), is located at amino acids 414-424 ([SEQ ID NO: 7]; Figure 5 a). This diacylglycerol binding motif is also found in the subsequently published *B. napus* DGAT sequences (Nikyiforuk et al, 1999;  
30 GenBank /EMBL Accession Nos. AF155224, SEQ ID NO:8; AF164434, SEQ ID NO:9). Among other cloned acyltransferases (e.g. GPATs, LPATs, dihydroxyacetone phosphate acyltransferases) it has been reported that there is an invariant proline in a highly hydrophobic block IV that may participate in acyl-CoA

binding (Lewin et al., 1999). In the TAG1 sequence, the hydrophobic block from residues 221-229 containing an invariant proline at residue 224, might constitute such a motif.

TAG1 showed some sequence similarity to other acyl CoA: cholesterol acyltransferases from a number of species (Chang et al., 1997). However, the similarity is largely confined to the C-terminus and is lower (around 30%) than is the similarity of TAG1 to the mammalian DGAT.

The TAG1 protein has multiple hydrophobic domains (Figure 5b) and an analysis by the PC Gene program predicted that the protein has 5 possible transmembrane segments (amino acids 178-195, 233-253, 363-388, 433-476, 486-507). In the mammalian DGAT, a putative tyrosine phosphorylation motif was observed (Cases et al., 1998), but no apparent tyrosine phosphorylation site could be found in TAG1. However, a visual examination revealed a consensus sequence (X-L<sup>200</sup>-X-K<sup>202</sup>-X-X-S<sup>205</sup>-X-X-X-V<sup>209</sup>; SEQ ID NO:10) identified as a targeting motif typical of members of the SnRK1 protein kinase family, with serine residue 205 being the residue for phosphorylation. The SnRK1 (SNF1-related protein kinase-1) proteins are a class of Ser/Thr protein kinases that have been increasingly implicated in the global regulation of carbon metabolism in plants (Hatfield and Hardie, 1998). This consensus SnRK1 targeting motif is also found in the subsequently published *B. napus* DGAT sequences (Nikyiforuk et al, 1999; GenBank /EMBL Accession Nos. AF155224; AF164434). Interestingly, similar SnRK1 targeting motifs could also be identified in the lyso-phosphatidic acid acyltransferases (LPATs) from coconut (Knutzon et al., 1995) and meadowfoam (Lassner et al., 1995), respectively.

25 *The TAG1 gene is ubiquitously expressed in Arabidopsis*

Northern blot analyses were performed to investigate the expression profile of the TAG1 gene. Total RNA was extracted from different tissues, including roots, leaves, flowers, developing siliques, young seedlings and germinating seeds. The highest steady-state level accumulation of TAG1 transcript was in RNA isolated from germinating seeds and young seedlings (Figure 6a). TAG1 transcripts were also detected in root, leaf and flower tissues, albeit at lower levels. Surprisingly, the TAG1 gene is expressed in developing siliques at a level that is comparable to that

of other vegetative tissues, but lower than that of germinating seeds and young seedlings. This expression profile in general is not inconsistent with the notion that DGAT is present in all plant tissues capable of TAG biosynthesis (Kwanyuan and Wilson, 1986). It has been shown in a number of plant species, including soybean 5 and safflower, that germinating seeds actively synthesize TAGs (Ichihara and Noda, 1981; Kwanyuan and Wilson, 1986; Wilson and Kwanyuan, 1986). The relatively high level of expression in roots is also consistent with the fact that root plastids are capable of synthesizing large amounts of triacylglycerol (Sparace et al., 1992).

Southern blot hybridization (Southern, 1975) was performed with genomic 10 DNA digested with several restriction enzymes including BgIII, EcoRI and HindIII. The *TAG1* gene has no internal BgIII and HindIII site, while one internal EcoRI site exists. Our Southern analysis suggested that *TAG1* most likely represents a single copy gene in the *Arabidopsis* genome (Figure 6b).

*An insertion mutation is found in the TAG1 gene in mutant AS11*

15 Alignment of the genomic sequence (accession no. AC003058; SEQ ID NO:3) with that of the *TAG1* cDNA [SEQ ID NO:1] revealed that the *TAG1* gene contains 16 exons and 15 introns, spanning a region of about 3.4 kb (Figure 7a). DNA containing the *TAG1* allele from AS11 was PCR-amplified and completely sequenced. The AS11 *TAG1* allele has a 147-bp insertion located at the central 20 region of intron 2. The insertion is a duplication of a segment that is composed of 12 bp from the 3' end of intron 1, the entire sequence of exon 2 (81 bp) and 54 bp from the 5' end of intron 2 (Figure 7a).

In order to rule out the possibility of PCR artifacts, two sets of primers were used to perform further PCR amplifications. Primers A and B (see Experimental 25 Procedures, *Primer Strategy*) located in exons 1 and 3, respectively, amplified a DNA fragment that is about 150 bp longer from AS11 (Figure 7b, lane 2) than that from the wild type (Figure 7b, lane 1). The second pair of primers, C and B (Experimental Procedures), with one to be found in both exon 2 and the insertion segment, and the other located in exon 3, generated two amplified fragments from 30 AS11 (Figure 7b, lane 4), while only one from the wild type (Figure 7b, lane 3). Hence these results confirmed that the insertion mutation the inventors identified

through sequencing, reflected the true nature of the mutation in the *TAG1* gene in the AS11 genome.

*The AS11 TAG1 transcript has an 81-bp insertion in its open reading frame*

Northern blot analyses indicated that there was no difference in the

- 5 expression profiles of the *TAG1* gene, between the AS11 mutant and wild type *A. thaliana*. In order to investigate the effect of the mutation at the transcript level, reverse-transcription PCR (RT-PCR) was performed to amplify the *TAG1* transcript from RNA extracted from germinating seedlings of mutant AS11. Sequencing analysis revealed that there is an 81-bp insertion composed entirely of exon 2 in the  
10 transcript from AS11. The exon 2 in the repeat is properly spliced. The alteration of the transcript thus does not disturb the reading frame. However, this additional exon 2 sequence in the AS11 transcript would result in an altered DGAT protein with the 27 amino acid insertion <sup>131</sup>SHAGLFNLCVVVLIAVNSRLIIENLMK<sup>157</sup> [SEQ ID NO:11]. The inventors' data shows that this insertion results in a 40-70% reduction  
15 in DGAT activity throughout seed development (Katavic et al., 1995). The 81 bp insert responsible for reduced DGAT activity in AS11 is visible in the comparison of RT-PCR products (Figure 7b: Compare lane 5 (WT) and lane 6 (AS11).) The DNA aberration observed in the AS11 mutant was unexpected, since ethyl methanesulfonate (EMS) generally causes point mutations. Although we cannot  
20 rule out the possibility that this AS11 mutant was the result of a spontaneous mutation event, EMS-induced deletions and insertions have been reported in other systems (Mogami et al., 1986, Okagaki et al., 1991)

*The TAG1 gene insertion in Arabidopsis mutant AS11 affects seed triacylglycerol accumulation, but not sterol ester accumulation in seeds.*

- 25 Because *TAG1* also showed some sequence homology to acyl CoA: cholesterol acyltransferases (ACATs) from a number of species (Chang et al., 1997), the inventors compared both triacylglycerol and sterol ester accumulation in seeds of the wild-type *A. thaliana* and AS11 mutant. While the triacylglycerol content and TAG/DAG ratios were reduced in AS11 (i.e. increased proportion of  
30 seed oil DAGs,) in contrast, the proportions of sterol esters in WT and AS11 seeds were similar, at 0.8 and 1% of the total lipid extract, respectively (Table 1). If the

*TAG1* lesion affected ACAT-like activity, one might expect a reduction in seed sterol esters, but this was not observed. These results indicated that *TAG1* is not involved in sterol-ester homeogenesis, and thus not an acyl CoA: sterol acyltransferase.

*TAG1 expression in yeast.*

- 5        The *TAG1* cDNA overexpressed in yeast resulted in a 3.5 to 4-fold increase in microsomal DGAT activity compared to plasmid only (*pYES2*) control transformants (Fig. 8), confirming that the *TAG1* gene product functions as a DGAT. When <sup>14</sup>C18:1-CoA was added to the yeast lysates, sterol esters were also labeled *in vitro* (data not shown), but there was no significant difference in the <sup>14</sup>C-labeled  
10      sterol esters produced by lysates from the *pYES2* GAL-induced control and the *pYES2:TAG1* Gal-induced transformant. This confirms that the *TAG1* product does not encode an acyl-CoA: sterol acyltransferase (like ACAT).

*Complementation of the *A. thaliana* AS11 Mutant Line by Transformation with the DGAT cDNA.*

- 15       The cloned full-length DGAT cDNA was used as a template for PCR amplification with the primers DGATXbaI (CTAGTCTAGAATGGCGATTTGGA; SEQ IN NO: 12) and DGATXhoI (GCGCTCGAGTTCATGACATCGA; SEQ ID NO:13) to provide new restriction sites on each end of the sequence as described in Experimental Procedures. A 1.6kb fragment was excised by a XbaI/KpnI digestion  
20      and ligated into the corresponding sites of the pSE129 vector (provided by Dr. P. Covello, PBI/NRC). pSE129A is a vector derived from the plant transformation vector pRD400 (Datla et al. 1992). The vector pSE129A contains the seed-specific napin promoter and the nos terminator cloned into the EcoRI and HindIII sites of the pRD400 plasmid (Figure 9). Hence in the DGAT-pSE129A construct, the  
25      *Arabidopsis* DGAT cDNA is under the control of the napin promoter. The construct integrity was confirmed by sequencing.

- The pSE129A containing the napin:DGATcDNA was introduced into *A. tumefaciens*, used to transform *A. thaliana* mutant AS11, and progeny analyzed as described in Experimental Procedures. A number of T<sub>2</sub> transgenic lines were  
30      isolated which complemented the fatty acid mutant phenotype found in AS11 (reduced 20:1 and elevated polyunsaturated C<sub>18</sub>s), restoring the wild-type seed fatty

acid profile (Figure 10). This finding confirms the nature of the lesion in AS11 and directly ties the AS11 lipid phenotype to this mutation.

*Over-Expression of the DGAT cDNA in Wild-Type *A. thaliana**

The cloned full-length DGAT cDNA was used as a template for PCR

- 5 amplification with the primers DGATXbaI (CTAGTCTAGAATGGCGATTTGGA ; SEQ ID NO:12) and DGATXhol (GCGCTCGAGTTCATGACATCGA; SEQ ID NO:13) to provide new restriction sites on each end of the sequence as described in Experimental Procedures. A 1.6kb fragment was excised by a XbaI/KpnI digestion and ligated into the corresponding sites of the pSE129 vector (provided by Dr. P. Covello, PBI/NRC).
- 10 pSE129A is a vector derived from the plant transformation vector pRD400 (Datla et al. 1992). The vector pSE129A contains the seed-specific napin promoter and the nos terminator cloned into the EcoRI and HindIII sites of the pRD400 plasmid (Figure 9). Hence in the DGAT-pSE129A construct, the
- 15 Arabidopsis DGAT cDNA is under the control of the napin promoter. The construct integrity was confirmed by sequencing.

- The pSE129A containing the napin:DGATcDNA was introduced into *A. tumefaciens*, used to transform wild-type *A. thaliana*, and progeny analyzed as described in Experimental Procedures. A number of T<sub>2</sub> transgenic lines were isolated which exhibited an increased oil content (Figure 11) an increased average 100-seed weight (Figure 12) and a strong linear correlation between the two traits (Figure 13).

- In terms of fatty acyl composition, wild type lines containing over-expressed DGAT cDNA showed a decrease in the total saturates, and increases in the monounsaturates and in the 18:1/[18:2 + 18:3] index, as shown in Table 2 below.
- 25 Such changes are all towards a 'healthier' oil profile, and can be applied directly to canola, other oilseeds in the *Brassicaceae* and other edible oil crops to produce similar oil composition improvements.

Table 2

Fatty acid composition of seed oil from *A. thaliana* non-transformed wild-type controls (WT Con) and three T2 transgenic lines (2', 9 and 11) of wild type transformed with the DGAT cDNA under the control of a napin promoter (napin: DGAT).

Line	Total Saturates <sup>a</sup> Wt %	Monounsaturates <sup>b</sup> Wt %	18:1/[18:2 + 18:3] index <sup>c</sup>
WT Control	15.1 ± 0.1	36.7 ± 0.2	29.9 ± 0.6
2' napin:DGAT	13.4	38.6	34.1
9 napin:DGAT	13.1	39.3	35.6
11 napin:DGAT	13.1	38.3	33.0

<sup>a</sup> Includes 16:0, 18:0, 20:0, 24:0

<sup>b</sup> includes 18:1, 20:1, 22:1, 24:1

<sup>c</sup> ([Wt % 18:1] + [Wt % 18:2 + Wt % 18:3]) × 100

## EXPERIMENTAL PROCEDURES

### *Plant Material*

5        *Arabidopsis thaliana* ecotype Columbia and mutant AS11 were grown under conditions described previously (Katavic et al., 1995). The *A. thaliana* mutant line AS11 was generated and characterized relative to wild type (WT) *A. thaliana* ecotype Columbia, as described by Katavic et al., (1995); (ATCC NO: PTA-1013).

### *DNA manipulation*

10      Standard methods and procedures were used for DNA preparation, plasmid propagation and isolation (Sambrook et al., 1989). Sequencing was conducted on an Applied Biosystems Model 373A DNA Sequencing System using the *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, Inc.). The nucleotide and the deduced amino acid sequences were compared with sequences 15 available in databanks using the BLAST program (Altschul et al., 1990).

*Southern and Northern analysis*

Total RNA was extracted from different tissues at various developmental stages, using the method of Lindstrom and Vodkin (1991). RNA samples were denatured with formaldehyde and separated on 1.2% formaldehyde-agarose gels.

- 5 About 5 µg of total RNA was loaded, and the amount of RNA per lane was calibrated by the ethidium bromide-staining intensity of the rRNA bands. Genomic DNA was isolated, digested with restriction enzymes and a Southern blot analysis was performed according to Sambrook et al. (1989). The TAG1 DNA probe was  $^{32}\text{P}$  labeled by random-priming according to protocols of the manufacture (BRL).

**10 PCR strategy**

Primers used for the amplification of the TAG1 gene were as follows:

- DGAT1 (AGACACGAATCCCATTCCACCGA; SEQ ID NO:14), DGAT2  
(AGTGGTGACAACGCAGGGATGATG; SEQ ID NO:15), DGAT3 (ATGGTCGCTCCCACATTGTGT;  
SEQ ID NO:16), DGAT4 (CATACAATCCCCATGACATTTATCA; SEQ ID NO:17). DGAT1  
15 and DGAT2 amplify the 5' half of the TAG1 gene and DGAT3 and DGAT4 amplify  
the 3' end of the TAG1 gene. Genomic DNA from AS11 was used as template for  
PCR amplification of the mutant TAG1 allele using the thermal profile: 94° C 3 min;  
40 cycles of 94° C 30 seconds, 62° C 45 seconds, 72° C 1 min; and 72° C 15 min.  
To further confirm the mutation, primers A (CGACCGTCGGTCCAGCTCATCGC; [SEQ ID  
20 NO:18]) and B (CGGCCCAATCTCGCAGCGATCTTC; [SEQ ID NO:19]), as well as primers C  
(TAAACAGTAGACTCATCATCG; [SEQ ID NO:20]) and D, were used in pairs, respectively,  
to amplify the internal fragment containing the mutation. The primers DGAT1 and  
DGAT4 were used for PCR amplification of the cDNA with an *A. thaliana* silique  
cDNA library as template. Primers A and B were also used in RT- PCR  
25 amplification of the cDNA fragment encompassing the insertion segment.

*Construction of TAG1 Multicopy Vector and Transformation and  
Characterization of DGAT Expression in Yeast*

- The TAG1 cDNA was cloned into pBluescript SK as described (Hadjeb and Berkowitz, 1996). The cDNA was cut out from the vector with KpnI/XbaI, and  
30 subsequently cloned into the respective sites of the yeast expression vector pYes2  
(Invitrogen). The construct was confirmed by sequencing. Constructs with TAG1

transcription under the control of the GAL1 promoter released a fragment of approximately 1.9 kb. Because the TAG1 fragment has its own initiating ATG codon, the product expressed is not a fusion protein. As a host for yeast expression, an SLC deletion strain (YMN5 [slc1Δ2::LEU2 ura3 ]) (kindly provided by M.M.

- 5 Nagiec and R.C. Dickson, University of Kentucky, Lexington, KY; Nagiec et al., 1993) was used; we reasoned that in this mutant, the endogenous DAG pool may be lower than in WT yeast, and that this would allow us to maximize the activity from over-expressed TAG1 in the presence of exogenously supplied <sup>14</sup>C-DAG during in vitro DGAT assays of transformant lysates. Yeast transformation was
- 10 performed according to Elble (1992). YMN5 transformants containing vector only (pYES2) were used as controls. Single colonies were cultured overnight in 20 mL of SD medium (Synthetic Dextrose medium with glucose and without uracil, as described by Ausubel et al., 1995, Vol. 2, p. 13.1.3) on a rotary shaker (270 rpm) at 28°C. Cells were pelleted from the overnight culture and resuspended in 50 mL of
- 15 medium for induction of expression (SD medium containing galactose and without uracil). Cells were reincubated at 28°C, with shaking at 270 rpm, and harvested after 4-6 hr. GAL-induced yeast transformants were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 100 mM Hepes-NaOH, pH 7.4, containing 1 mM EDTA and 1 mM DTT. Cell lysates were prepared using acid-washed glass
- 20 beads as described by Ausubel et al. (1995). Protein in yeast lysates was measured using the Bradford (1976) assay, protein levels in each lysate were normalized and aliquots (250 µg protein) were assayed for DGAT activity as described below.

#### *Lipid Substrates and DGAT Analyses*

- 25 <sup>14</sup>C-labeled diolein [1-<sup>14</sup>C oleic] (Sp. activity 55 mCi/ mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The <sup>14</sup>C-labelled *sn*-1,2-diolein isomer was purified by TLC on borate-impregnated plates and emulsified in Hepes buffer the presence of 0.2% Tween-20 as described by Taylor et al., (1991). 20:1-CoA, CoASH, ATP, and all other biochemicals were purchased from Sigma.

- 30 DGAT assays were conducted at pH 7.4, with shaking at 100 rpm in a water bath at 30°C for 30-60 min. Assay mixtures (0.5 mL final volume) contained lysate protein (250 µg), 90 mM Hepes-NaOH, 0.5 mM ATP, 0.5 mM CoASH, 1 mM MgCl<sub>2</sub>, 200 µM *sn*-1,2 diolein (sp. activity 2 nC/nmol) in 0.02% Tween 20, and 18 µM 20:1-

CoA as the acyl donor. The <sup>14</sup>C-labeled TAGs were isolated by TLC and quantified as described by Taylor et al (1991).

*Further Lipid and Sterol Ester Analyses in AS11 and WT:*

- Total lipid extracts (TLEs), and lipid class analyses in WT and the AS11
- 5 mutant were performed as described by Taylor et al., (1991; 1992) and by Katavic et al., (1995). Relative seed oil content was also measured by magic angle sample spinning <sup>1</sup>H-NMR, according to the method of Rutar (1989). Analyses were conducted with 200-seed samples of intact wild-type and AS11 seeds using a Bruker AM wide-bore spectrometer (Bruker Analytische Masstechnik GHBH,
- 10 Silberstreifen D-76287, Rheinstetten4/Karlsruhe, Germany) operating at 360 MHz. To reduce anisotropic line broadening, the seed sample was rotated at 1 kHz in a zirconium rotor oriented 54.7° to the magnetic field. The integration response for resonances attributable to liquid-like oil were summed and the value for AS11 seed was recorded relative to the response for the WT control seed sample, the latter set
- 15 at a value of 1.00.

Sterol esters were purified from the TLEs by thin layer chromatography (TLC) on Silica H plates developed in hexane: diethyl ether: formic acid (80:20:2, v/v). After elution from the silica H with chloroform: methanol (2:1, v/v), the sterol esters were quantified by saponification followed by methylation of the resulting fatty acids with 3N methanolic-HCl. The fatty acid methyl esters (FAMEs) were analyzed by GC as described previously (Taylor et al., 1991). The free sterols released by saponification were also analyzed by GC on a 30 m DB-5 column; GC temperature program : initial temp: 180°C, increasing at 10°C/min to 300°C and held at this temperature for 15 min. The sterol ester content was reported as a % of the TLE;

20 25 i.e. FAMEs released from sterol esters calculated as proportion of the FAMEs released by transmethylation of the total lipid extract (TLE).

*Construction of Plant Transformation Vector Containing the Wild-Type DGAT gene for Over-Expression in WT A. thaliana and Complementation of the A. thaliana AS11 Mutant:*

Gen 1 (GAGAGGGATCCACGCTCACGACCCATTCTTCCCG; [SEQ ID NO:21]), and

Gen 2 (AAGAAGGATCCATCCCCAAAACGGGACCACAA; [SEQ ID NO:22])

- 5 were synthesized according to sequences upstream and downstream of the TAG1 gene. These primers were used to PCR amplify a genomic fragment of 5.1 kb from wild-type *A. thaliana*. The PCR fragment was purified and digested with BamHI and inserted into the corresponding site in plasmid pRD400 (Datla et al. 1992) to generate the plant transformation vector DGATg-pRD400.
- 10 **Construction of DGAT cDNA Plant Transformation Vector for Seed-Specific Expression:**

The cloned full-length DGAT cDNA was used as a template for PCR amplification with the primers DGATXbaI (CTAGTCTAGAACGGCGATTTGGA; SEQ ID NO:12) and DGATXhoI (GCGCTCGAGTTCATGACATCGA; SEQ ID NO: 13) to provide new restriction sites on each end of the sequence. The PCR profile was as follows: 94°C 1 min; 30 cycles of 94°C 30 seconds, 55°C 30 seconds, 72°C 1 min; and 72°C 5 min. The PCR product was then ligated into the PCR-2.1 vector (InVitrogen). A 1.6kb fragment was excised by a XbaI/KpnI digestion and ligated into the corresponding sites of the pSE129 vector (provided by Dr. P. Covello, 20 PBI/NRC). pSE129A is a vector derived from the plant transformation vector pRD400 (Datla et al. 1992). The vector pSE129A contains the seed-specific napin promoter and the nos terminator cloned into the EcoRI and HindIII sites of the pRD400 plasmid (See Figure 9). Hence in the DGAT-pSE129A construct, the *Arabidopsis* DGAT cDNA is under the control of the napin promoter. The construct 25 integrity was confirmed by sequencing.

**Transformation of Agrobacterium with Plant DGAT Vector Constructs:**

Electrocompetent *Agrobacterium* cells, GV3101 (pMP90) strain, were prepared as follows: An *Agrobacterium* culture was grown 24 to 48 hrs in 2YT, and when the absorbance at 600 nm reached 0.5 to 0.7, the cells were chilled on ice 30 and pelleted by centrifugation (5,000 x g, 10 min in a GSA rotor at 4°C). The pellet was washed in 1, 0.5 and 0.02 volumes of cold 10% sterile glycerol and

resuspended in 0.01 volume of cold 10% glycerol. The electrocompetent cells were then frozen in liquid N<sub>2</sub> and stored at -70°C. The *Agrobacterium* cells were

transformed by electroporation with 20-50 ng of transforming DNA (either DGATg-pRD400 or DGAT-pSE129A) according to the manufacturer's instructions, plated on

- 5 a selective medium (LB with 50 µg/mL kanamycin) and incubated overnight at 28°C. Single transformed cells were grown overnight (28°C, 225 r.p.m.) in 5 mL LB with 50 µg/mL Kanamycin and 25 µg/mL Gentamycin. DNA extraction and purification were performed with a Qiaprep Spin Miniprep kit (Qiagen). The fidelity of the construct was re-checked by DNA sequencing before plant transformation.

#### 10 Transformation of *Arabidopsis thaliana*:

The transformation protocol was adapted from that described by Clough and Bent (1998). Seeds of *Arabidopsis thaliana* ecotype Columbia and mutant AS11 (Katavic et al., 1995) were grown at 22°C under fluorescent illumination (120 µE·m<sup>-2</sup>·s<sup>-1</sup>) in a 16 h light/8 hour dark regime. Typically, four to six plants were raised in a

- 15 10 cm<sup>2</sup> pot in moistened Terra-lite Redi-earth (W. R. Grace & Co. Canada Ltd. Ajax, ON, Canada). To prevent the soil mix in the pot from falling into the inoculation media, soil was mounded as a platform with seeds sown on top, and the whole pot covered by a nylon window screen and secured by a rubber band. Plants were vacuum infiltrated in an *Agrobacterium* suspension when the first flowers started
- 20 opening.

To grow *Agrobacterium*, a 5 mL suspension in LB medium containing 50 µg/mL kanamycin and 25 µg/mL gentamycin was cultured overnight at 28°C. The day before infiltration, this "seed culture" was divided into four flasks containing 250 mL of LB medium supplemented with 50 µg/mL kanamycin and 25 µg/mL

- 25 gentamycin. These culture were grown overnight at 28°C. The next morning after the absorbance at 600 nm was checked (approximately = 1.0), the cells were harvested by centrifugation (5,000 x g, 10 min in a GSA rotor at room temperature) and resuspended in the infiltration medium (sucrose 5%; Silwet-77 0.005% in water) to obtain an optical density at 600 nm of 0.8. The *Agrobacterium* suspension was
- 30 then poured into a beaker and the potted plants inverted into the beaker so that the flowers and bolts were submerged. The beaker was then placed into a large Bell jar and a vacuum drawn using a vacuum pump, until bubbles formed on the stem

surfaces and the solution started to bubble slightly, and then the vacuum was released rapidly. [Note: The necessary time and pressure will vary from one lab setup to the next, but good infiltration is visibly apparent as uniformly darkened, water-soaked tissue.] Pots were removed from the beaker, laid on their side in a

- 5 plastic tray and covered with a plastic dome, to maintain humidity. The following day, the plants were uncovered, set upright and allowed to grow for approximately four weeks in a growth chamber under continuous light conditions as described by Katavic et al., (1995). When the siliques were mature and dry, seeds were harvested and selected for positive transformants.

- 10 **Selection of Putative Transformants (Transgenic plants) and Analysis of Transgenic Plants:**

For each construct, seeds were harvested in bulk. Seeds were surface-sterilized by submerging them in a solution containing 20% bleach and 0.01% Triton X-100 for 20 min, followed by three rinses with sterile water. Sterilized seeds were

- 15 then plated by resuspending them in sterile 0.1% phytagar at room temperature (about 1 mL phytagar for every 500-1000 seeds), and then applying a volume containing 2,000-4,000 seeds onto 150 x 15 mm kanamycin selection plate. Plates were incubated for 2 days in the cold without light, and then grown for 7-10 days in a controlled environment (22°C under fluorescent illumination (120  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) in a
- 20 16 h light/8 hour dark regime). The selection media contain ½ MSG medium, 0.8% phytagar, 3% sucrose, 50ug/mL kanamycin and 50ug/ mL Timentin. Petri dishes and lids were sealed with a Micropore™ surgical tape tape (3M Canada, London, ON, Canada). After 7-10 days, drug-resistant plants that had green leaves and well established roots within the medium were identified as transformants and at the 3-5
- 25 leaf stage, selected transformants were transplanted into flats filled with heavily moistened soil mix. Transformants were grown to maturity and mature seeds ( $T_2$  generation as defined in Katavic et al., (1994)) were harvested from individual plants, and further analyzed.

**DNA isolation from and analysis of Transformants**

- 30 Genomic DNA was isolated from individual  $T_1$  plants following the protocol of Dellaporta et al., (1983). A PCR amplification using the paired primers described previously for the DGAT cDNA or for the DGAT gene, was performed to confirm the

presence of the cDNA or the gene, respectively, in the T<sub>1</sub> transformants. Southern analyses (Southern, 1975) were performed to select the transformants containing a single copy of the inserted fragment. DNA samples were digested with restriction enzymes (Bgl II for the DGAT cDNA and Eco RI for the DGAT gene), resolved by

- 5 electrophoresis on a 1% agarose gel, and Southern blotting performed using a nylon filter (Hybond-N+, Amersham) according to Sambrook *et al.* (1989). The DGAT cDNA fragment, labelled with  $\alpha$ -[<sup>32</sup>P] dCTP (NEN/DuPont) using the Random Primer DNA labelling kit (Gibco BRL), was used as a probe. Hybridization was performed at 60°C according to Church and Gilbert (1984). The filter was then  
10 exposed to Kodak X-OMAT-AR film.

#### DEPOSIT INFORMATION

The following biological material has been deposited at the American Type Culture Collection (ATCC) of 10801 University Boulevard, Manassas, Virginia, 20110-2209, U.S.A. All of these deposits were made on behalf of the

- 15 Applicant/Assignee (National Research Council of Canada) under the terms of the Budapest Treaty on the dates indicated, and have been given the accession numbers shown below.

	Deposited Material	Date of Deposit	Accession No.
	Arabidopsis DGAT gene	November 29, 1999	PTA-988
20	Arabidopsis DGAT cDNA	November 29, 1999	PTA-989
	Arabidopsis ASII seeds	December 3, 1999	PTA-1013

The deposit receipts are shown later in this description.

#### 25 SEQUENCE LISTING FREE TEXT

The Sequence Listing provided below contains free text entries in respect of SEQ ID NOs: 12 to 22. The free text used in the Sequence Listing is repeated as follows:

- SEQ ID NO:12      Primer of DGATXbal  
30      SEQ ID NO:13      Primer of DGATXhol

	SEQ ID NO:14	Primer DGAT1
	SEQ ID NO:15	Primer DGAT2
	SEQ ID NO:16	Primer DGAT3
	SEQ ID NO:17	Primer DGAT 4
5	SEQ ID NO:18	Primer A
	SEQ ID NO:19	Primer B
	SEQ ID NO:20	Primer C
	SEQ ID NO:21	Primer Gen 1
	SEQ ID NO:22	Primer Gen 2.

10

A summary of all of the listed sequences is provided below for ease of review:

SEQ ID NO:1 - (pDGAT; vector containing isolated and purified deoxyribonucleic acid cDNA; ATCC No PTA-989), Genbank/EMBL Accession No. AJ238008.

15 SEQ ID NO:2 - The deduced amino acid sequence of the *Arabidopsis* DGAT (AtTAG1) protein.

SEQ ID NO:3 - (pgenomic DGAT; vector containing isolated and purified genomic deoxyribonucleic acid (genomic DNA) ATCC No PTA-988).

20 SEQ ID NO:4 - MDGAT, mouse DGAT [GenBank/EMBL Accession No. AF078752 (Cases et al., 1998)].

SEQ ID NO:5 - HARGP1, human ARGP1 protein [GenBank/EMBL Accession No. AF059202; Oelkers et al., 1998].

SEQ NO:6 - *Arabidopsis thaliana* expressed sequence tag (EST) [accession no. AA042298].

25 SEQ ID NO:7 - A diacylglycerol/phorbol ester-binding motif found in SEQ ID NO:2, SEQ ID NO:8 and SEQ ID NO:9 ( <sup>414</sup>HKWMVRHIYFP<sup>424</sup> ).

SEQ ID NO:8 - *B. napus* DGAT amino acid sequence GenBank EMBL Accession No AF155224.

SEQ ID NO:9 - *B. napus* DGAT amino acid sequence GenBank/EMBL Accession No. AF164434.

SEQ ID NO: 10 - Targeting motif typical of members of the SnRK1 protein kinase family found in SEQ ID NO:2, SEQ ID NO:8 and SEQ ID NO:9 X-L<sup>200</sup>-X-K<sup>202</sup>-  
5 X-X-S<sup>205</sup>-X-X-X-V<sup>209</sup>

SEQ ID NO:11 - A 27 amino acid insertion repeat in SEQ ID NO:2 found in the *Arabidopsis thaliana* AS11 mutant.

<sup>131</sup>SHAGLFNLCVVVLIAVNSRLIIENLMK<sup>157</sup>

SEQ IN NO: 12 - CTAGTCTAGAACGGCGATTTGGA ( nucleotide sequence of  
10 Primer DGATXbaI ).

SEQ ID NO:13 - GCGCTCGAGTTCATGACATCGA ( nucleotide sequence of  
Primer DGATXhol).

SEQ ID NO:14 - AGACACGAATCCCATTCCCACCGA ( nucleotide sequence of  
Primer DGAT1).

15 SEQ ID NO:15 - AGTGGTGACAACGCAGGGATGATG ( nucleotide sequence of  
Primer DGAT2).

SEQ ID NO:16 - ATGGTCGCTCCCACATTGTGT ( nucleotide sequence of Primer  
DGAT3).

20 SEQ ID NO:17 - CATAACAATCCCCATGACATTATCA ( nucleotide sequence of  
Primer DGAT4).

SEQ ID NO:18 - CGACCGTGGTTCCAGCTCATCGG ( nucleotide sequence of  
Primer A).

SEQ ID NO:19 - GCGGCCAATCTCGCAGCGATCTTG (nucleotide sequence of  
Primer B).

25 SEQ ID NO:20 - TAAACAGTAGACTCATCATCG (nucleotide sequence of  
Primer C).

SEQ ID NO:21 - GAGAGGATCCACGCTCACGACCCATTCTCCG (nucleotide sequence of primer Gen 1).

SEQ ID NO:22 - AAGAAGGATCCATCCCCAAAACGGGACCACCAA  
(nucleotide sequence of primer Gen 2).

5 SEQ ID NO:23 - AS11 mutant DGAT cDNA nucleotide sequence.

SEQ ID NO:24 - AS11 mutant DGAT genomic DNA nucleotide sequence.

SEQ ID NO:25 - the deduced amino acid sequence of SEQ ID NO:23.

# ATCC

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**BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF  
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE**

**INTERNATIONAL FORM**

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AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2**

To: (Name and Address of Depositor or Attorney)

NRC Canada  
Attn: David C. Taylor  
Plant Biotechnology Institute  
110 Gymnasium Place  
Saskatoon, SK S7N 0W9 Canada

Deposited on Behalf of: National Research Council of Canada

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Identification Reference by Depositor: Patent Deposit Designation

<i>Arabidopsis</i> DGAT gene	PTA-988
<i>Arabidopsis</i> DGAT cDNA	PTA-989

The deposits were accompanied by:  a scientific description  a proposed taxonomic description indicated above. The deposits were received November 29, 1999 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:  We will not inform you of requests for the strains.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

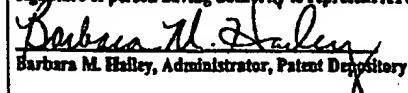
If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

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Barbara M. Hafley, Administrator, Patent Depository

Date: December 3, 1999

cc: Kirby, Eades, Gale and Baker

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To: (Name and Address of Depositor or Attorney)

National Research Council of Canada  
Attn: David C. Taylor  
Plant Biotechnology Institute  
110 Gymnasium Place  
Saskatoon, SK S7N 0W9  
Canada

Deposited on Behalf of: National Research Council of Canada

Identification Reference by Depositor:

Patent Deposit Designation

Arbitrarily AS11

PTA-1013

The seeds were accompanied by:  a scientific description  a proposed taxonomic description indicated above.  
The seeds were received December 3, 1999 by this International Depository Authority and have been accepted.

AT YOUR REQUEST:  We will not inform you of requests for the seeds..

The seeds will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the seeds and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said seeds.

If the seeds should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with viable seeds of the same.

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International Depository Authority: American Type Culture Collection, Manassas, VA 20110-2209 USA.

Signature of person having authority to represent ATCC:

  
Barbara M. Hailey, Administrator, Patent Depository

Date: January 3, 2000

cc: Kirby, Eades et al.

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The teachings of the above references are hereby incorporated by reference.

CLAIMS:

1. Isolated and purified deoxyribonucleic acid (DNA), characterized in that said DNA includes a sequence according to SEQ ID NO:1 or a part of SEQ ID NO:1, or a sequence that is substantially homologous to SEQ ID NO:1.
2. Isolated and purified deoxyribonucleic acid (DNA), characterized in that said DNA includes a sequence according to SEQ ID NO:3 or a part of SEQ ID NO:3, or a sequence that is substantially homologous to SEQ ID NO:3.
3. A vector for transformation of plant cells, characterized in that said vector contains a deoxyribonucleic acid sequence according to SEQ ID NO:1, or a part of SEQ ID NO:1, or a sequence that is substantially homologous to SEQ ID NO:1.
4. A vector for transformation of plant cells, characterized in that said vector contains a deoxyribonucleic acid sequence according to SEQ ID NO:3, or a part of SEQ ID NO:3, or a sequence that is substantially homologous to SEQ ID NO:3.
5. A vector for transformation of plant cells, characterized in that said vector contains a deoxyribonucleic acid sequence according to SEQ ID NO:23, which is SEQ ID NO: 1 altered to contain an 81 bp insertion, such that the deduced amino acid sequence of the encoded protein contains the repeated sequence SHAGLFNLVVLIAVNSRLIIENLMK according to SEQ ID NO:25, where the spacing and identity of the underlined amino acids are identical or are replaced by conserved substitutions.
6. A vector according to claim 3 or claim 5, characterized in that said sequence is present in said vector in a sense orientation.
7. A vector according to claim 3, characterized in that said sequence is present in said vector in an anti-sense orientation.
8. Plasmid pDGATcDNA (ATCC PTA-989).
9. Plasmid pDGATgene (ATCC PTA-988).

10. A plant having a genome, characterized in that the genome contains an introduced nucleotide sequence of SEQ ID NO:1, or a part of SEQ ID NO:1, or a sequence that is substantially homologous to SEQ ID NO:1.

11. A plant seed having a genome, characterized in that said genome contains an introduced nucleotide sequence of SEQ ID NO:1, or a part of SEQ ID NO:1, or a sequence that is substantially homologous to SEQ ID NO:1.

12. A genetically transformed plant, characterized in that said genome has been transformed by a vector according to claim 3 or claim 4 or claim 5.

13. A genetically transformed plant seed, characterized in that said seed has been transformed by a vector according to claim 3 or claim 4 or claim 5.

14. A plant seed as claimed in Claim 11 or Claim 13, characterized by exhibiting an altered seed oil content compared to an average of a statistically-significant number of seeds of genetically-unmodified plants of the same genotype grown in identical conditions at the same time.

15. A plant seed as claimed in Claim 11 or Claim 13, characterized by exhibiting an altered diacylglycerol content in its seed oil compared to an average of a statistically-significant number of seeds of genetically-unmodified plants of the same genotype grown in identical conditions at the same time.

16. A plant seed as claimed in 11 or 13, characterized by exhibiting a seed oil with an altered fatty acyl composition compared to an average of a statistically-significant number of seeds a genetically-unmodified plantof the same genotype grown in identical conditions at the same time.

17. A plant as claimed in 10 or 12, characterized by exhibiting an enhanced biomass compared to an average of a statistically-significant number of genetically-unmodified plants of the same genotype grown in identical conditions at the same time.

18. A seed as claimed in 11 or 13, characterized by exhibiting an enhanced biomass compared to an average of a statistically-significant number of seeds of genetically-unmodified plants of the same genotype grown under identical conditions at the same time.

19. A method of producing transgenic plants by introducing a nucleotide sequence into a genome of said plant, characterized in that said nucleotide sequence introduced into said genome includes SEQ ID NO:1 or SEQ ID NO:3, or a part of SEQ ID NO:1 or SEQ ID NO:3, or a sequence that is substantially homologous to SEQ ID NO:1, or to SEQ ID NO:3, or to or SEQ ID NO: 1, or a part of SEQ ID NO:1 or SEQ ID NO:3, or a sequence that is substantially homologous to SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:1 containing an 81 bp insertion [SEQ ID NO:23] such that the deduced amino acid sequence of the encoded protein contains the repeated sequence SHAGLFNLCVVVLIAVNSRLIENLMK according to SED ID NO:25, where the spacing and identity of the underlined amino acids are identical or are replaced by conserved substitutions.

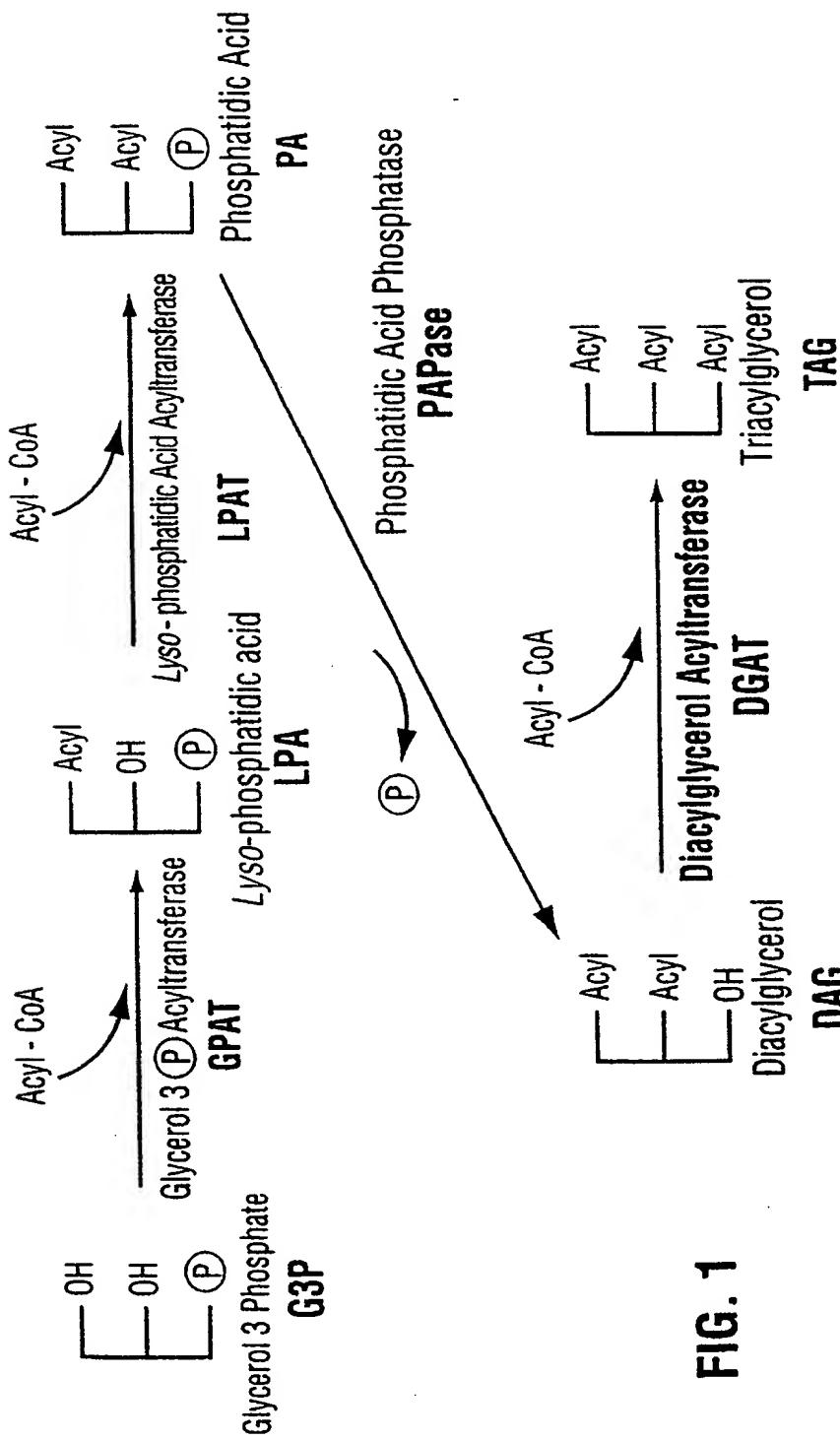
20. A method according to claim 19, characterized in that said plant is a member of the Brassicaceae.

21. A method according to claim 19, characterized in that said plant is selected from the group consisting of *Arabidopsis thaliana*, borage (*Borago* spp.), Canola, castor (*Ricinus communis*), cocoa bean (*Theobroma cacao*), corn (*Zea mays*), cotton (*Gossypium* spp.), *Crambe* spp., *Cuphea* spp., flax (*Linum* spp.), *Lesquerella* and *Limnanthes* spp., Linola, nasturtium (*Tropaeolum* spp.), *Oenothera* spp., olive (*Olea* spp.), palm (*Elaeis* spp.), peanut (*Arachis* spp.), rapeseed, safflower (*Carthamus* spp.), soybean (*Glycine* and *Soja* spp.), sunflower (*Helianthus* spp.), tobacco (*Nicotiana* spp.), *Vernonia* spp., wheat (*Triticum* spp.), barley (*Hordeum* spp.), rice (*Oryza* spp.), oat (*Avena* spp.) sorghum (*Sorghum* spp.), rye (*Secale* spp.) and other members of the Gramineae.

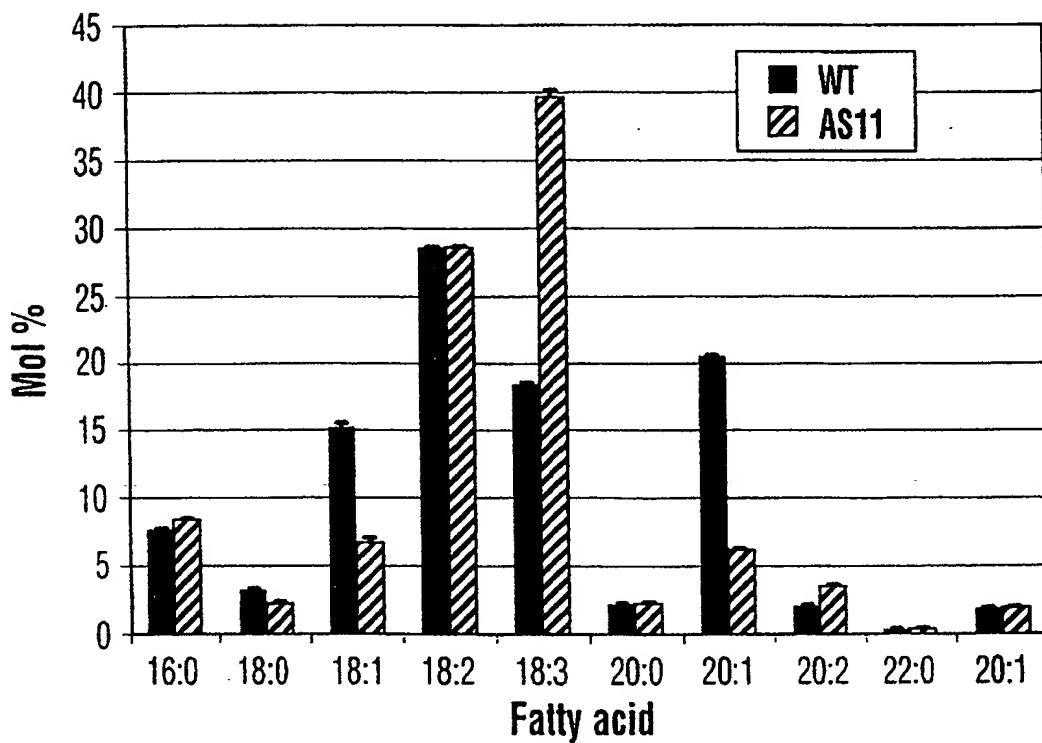
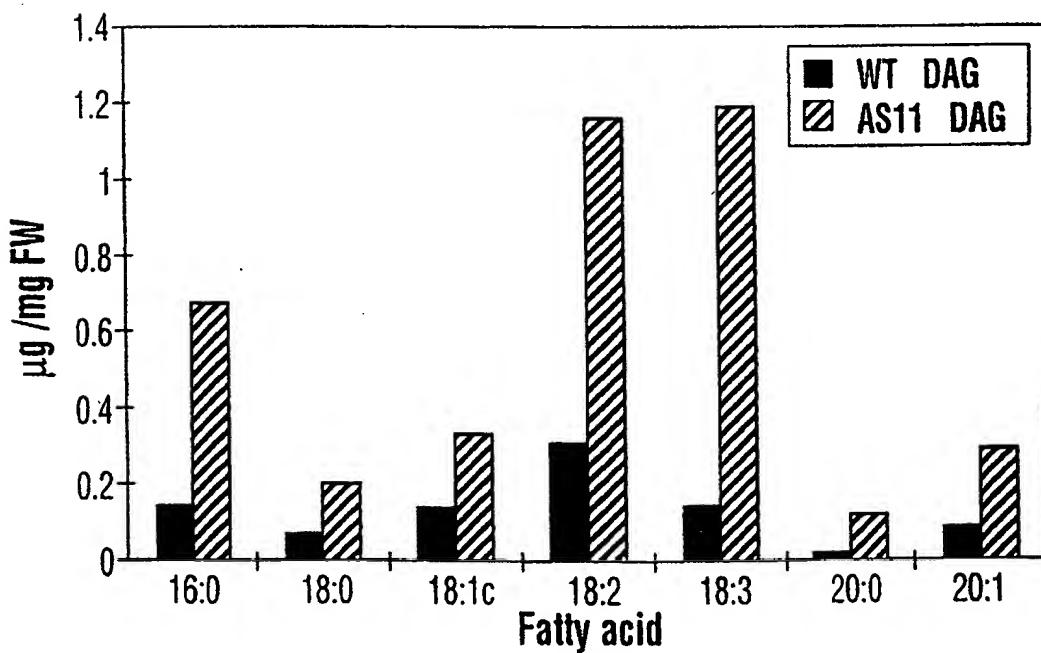
22. A plant DNA sequence or part thereof, characterized in that the sequence is substantially homologous to at least a part of SEQ ID NO:1 or SEQ ID NO:3, and in that said sequence has been isolated, characterized or designed using sequence information from SEQ ID NO:1 or SEQ ID NO:3, or SEQ ID NO:1 containing an 81 bp insertion [SEQ ID NO:23] such that the deduced amino acid sequence of the encoded protein contains the repeated sequence SHAGLFNLCVVVLIAVNSRLIENLMK according to SED ID NO:25, where the spacing and identity of the underlined amino acids are identical or are replaced by conserved substitutions.

23. A method of changing the oil content, acyl composition or diacylglycerol/triacylglycerol proportions of the seed oil of plant seeds by introducing a sense or anti-sense nucleic acid construct into a plant transformation vector, using the vector to transform the genome of a plant or plant seed, and then growing the plant or plant seed and extracting the oil from the plant seed, characterized in that said nucleic acid sequence is SEQ ID NO:1 or SEQ ID NO:3, or a part of SEQ ID NO:1 or SEQ ID NO:3, or a sequence that is substantially homologous to SEQ ID NO:1 or SEQ ID NO:3 or SEQ ID NO:1 [SEQ ID NO:23] containing an 81 bp insertion such that the deduced amino acid sequence of the encoded protein contains the repeated sequence SHAGLFNLCVVVLIAVNSRLIIENLMK according to SED ID NO:25, where the spacing and identity of the underlined amino acids are identical or are replaced by conserved substitutions.

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**FIG. 1**

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**FIG. 2****FIG. 3**

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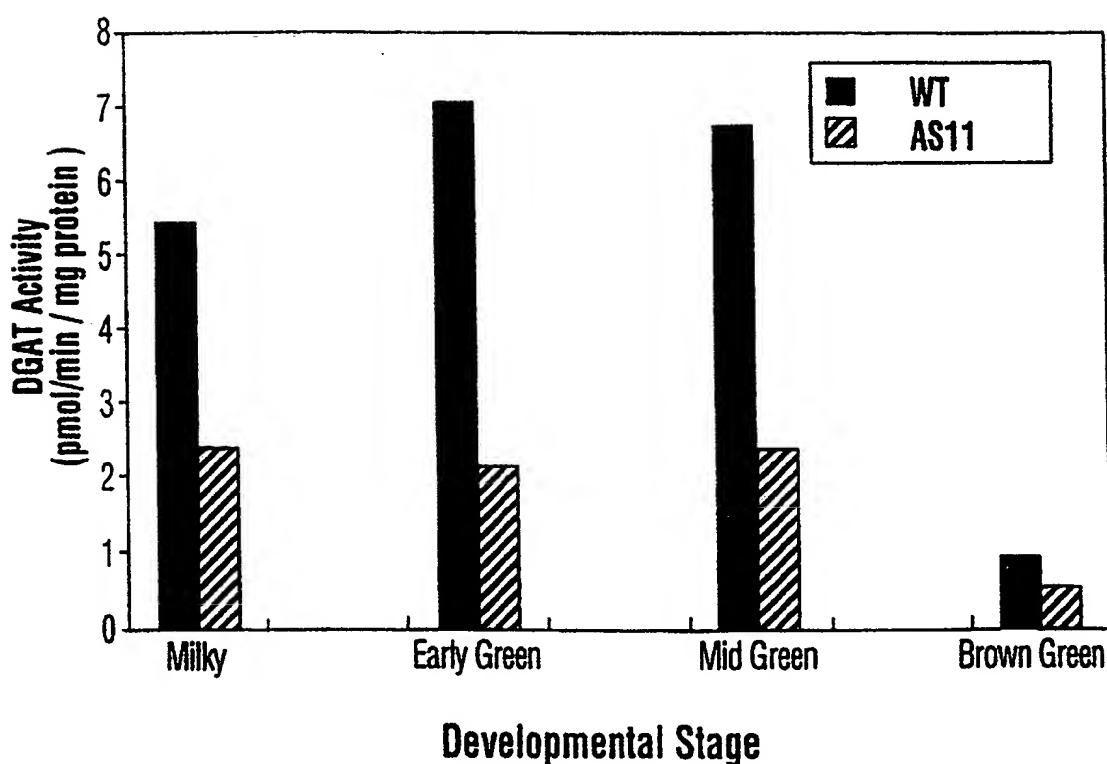


FIG. 4

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(a)

At TAG1	M A I L D S A C V T T V T E N G G E F V D L D R L R R R K S R S D S S N G L L L S G S D N N S P S	50
MDGAT	M G D R G G A G S S R . . . . .	RRR T G S R V S V Q G . . . . . G S G P K V E E
HARGP1	M G D R . . . G S S R . . . . .	RRR T G S R P S S H G . . . . . G G G P A A A E
At TAG1	D E V G A P A D V R D R I D S V V N D D A Q G T A N L A G D N N G G D N N G G G R G G G E C R G N	100
MDGAT	D E V R D A A V S P D L . . . . .	G A G G D A P A P A P A P A H T R D K D G R T S V G E G Y . . .
HARGP1	D E V R D A A A G P D V . . . . .	G A A G D A P A P A P . . . . . N K D G D A G V G S C H . . .
At TAG1	A D A T E T Y R P S V P A H R R A R E S P L S S D E I E K Q S H A G E N I C V V V L I A V N S R L	150
MDGAT	. . . . . D L R C . . . . . H R L Q D S L F S S D E G H S N Y R . G E L N W C V V V L I L S N V R L	112
HARGP1	. . . . . W E L R C . . . . . H R L Q D S L F S S D E G H S N Y R . G E L N W C V V V L I L S N V R L	103
At TAG1	I I E N I L K Y G W L I R T D F W P S S R S L D . . . W P L F M C . C I S L S I F P E A A F T V S	196
MDGAT	F F E N L K Y G I L V D P . I Q V V S L F L K D P Y S W P A . P C V I I A S N E F V V A A F O V E	160
HARGP1	F F E N L K Y G I L V D P . I Q V V S L F L K D P H S W P A . P C L V I I A A N Y F A V A A F O V E	151
At TAG1	K L A V L Q K Y I E P V V V F L H I I T M T E V L V P V Y V T L R C E S A F L S G V T I M I E C	246
MDGAT	K R E A V G A E I I E Q M G I E L L H V A N L A T I I C E P A A V A V L V E S I T P V G S V F A L A S Y	210
HARGP1	K R E A V G A E I I E Q A G E I L L H V A N L A T I I C E P A A V V L L V E S I T P V G S I I A L M A H	201
At TAG1	. I I I A L K L V S Y A H T S Y . . . . D I R S L A N A A D K . . . . . A N P E V S Y Y V S L . . .	282
MDGAT	S I I I A L K L V S Y R D V N L W C R Q R R W A K A V S T G K K V S G A A A Q Q A V S Y P D N L T Y	260
HARGP1	T I I I A L K L V S Y R D V N S W C . . . R R A R A K A A S A G K K A S S A A A P H I V S Y P D N L T Y	249
At TAG1	K S L A Y F M V A P T L C Y Q P S I P R S A C I R K G I W A R Q F A K T I I F T G F M G F I I E Q M	332
MDGAT	R D L Y Y F I F A P T L C Y E L N E P R S P R I R K R E E I I R R V L E M E F F T Q L Q V G E I Q Q N	310
HARGP1	R D L Y Y F I F A P T L C Y E L N E P R S P R I R K R E E I I R R I L E M E F F T Q L Q V G E I Q Q N	299
At TAG1	I N P I M R N S K H P L K G D I I Y A . . I E R E L K L I S V P N L Y I W L C M F Y C F F H L W L N I	380
MDGAT	M V P T T O N S M K P F K . D M D Y S R I I I E R E L K L I S V P N H I I W L I F F Y W F F H S C L N A	359
HARGP1	M V P T T O N S M K P F K . D M D Y S R I I I E R E L K L I S V P N H I I W L I F F Y W L F H S C L N A	348
At TAG1	E A E L I C F G D R E F Y I D W W N I I K S V G D I I R M M N N P P V H K W M I R H I Y F P C L R S K I	430
MDGAT	V A E L I I Q F G D R E F Y I D W W N I I S E S V T Y I I W Q N W N I P P V H K W C I R H F Y K P M L R H G S	409
HARGP1	V A E L I I Q F G D R E F Y I D W W N I I S E S V T Y I I W Q N W N I P P V H K W C I R H F Y K P M L R H G S	398
At TAG1	P K I T E A I I I A F L V S A V F H E L C G I V P C R E F F I L W A F L G I M F O D P L V . F I T N I L	479
MDGAT	S K W M A R T G V F L T S A F F H E Y L V S V P L R M F F I L W A F T A M M A C V P L A I W V G R E F	459
HARGP1	S K W M A R T G V F L A S A F F H E Y L V S V P L R M F F I L W A F T G V I M A C V P L A W F V G R E F	448
At TAG1	O E R E G S T V G N M I F F F I F C I F G Q F U C V L I P Y Y H D . . E M N R K G S M S . .	520
MDGAT	O G N Y G N A A . . . . V V V T L . I I G Q P F A V L M V V H D Y Y V V N Y D A P V G . V	498
HARGP1	O G N Y G N A A . . . . V V L S L . I I G Q P F A V L M V V H D Y Y V V N Y E A P A A E A	488

FIG. 5(a)

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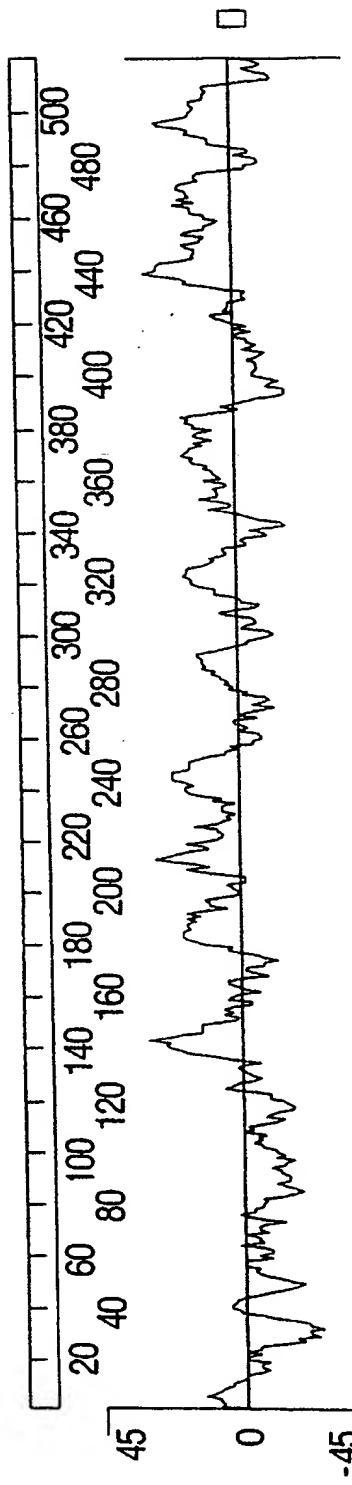


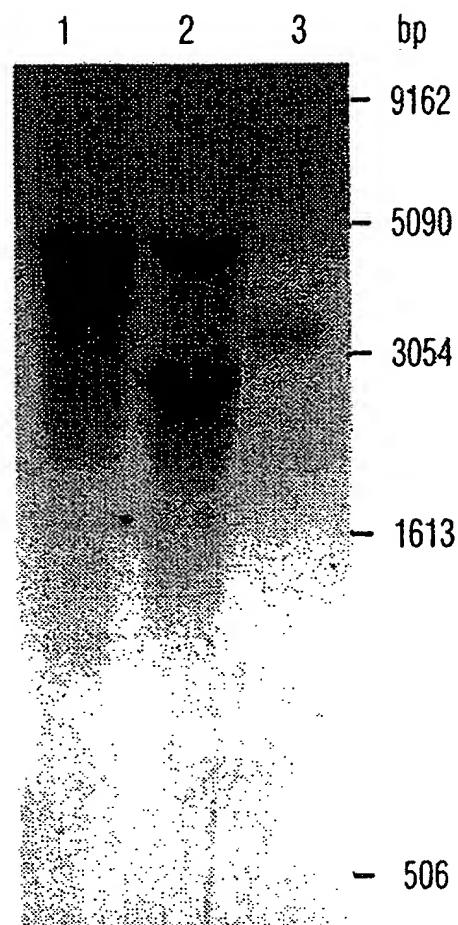
FIG. 5(b)

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RT LF FL YS SL GS

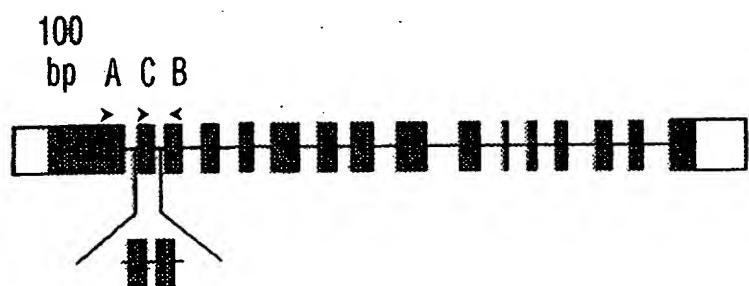


**FIG. 6(a)**

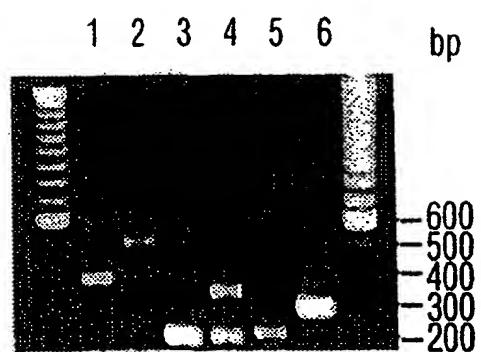


**FIG. 6(b)**

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**FIG. 7(a)**



**FIG. 7(b)**

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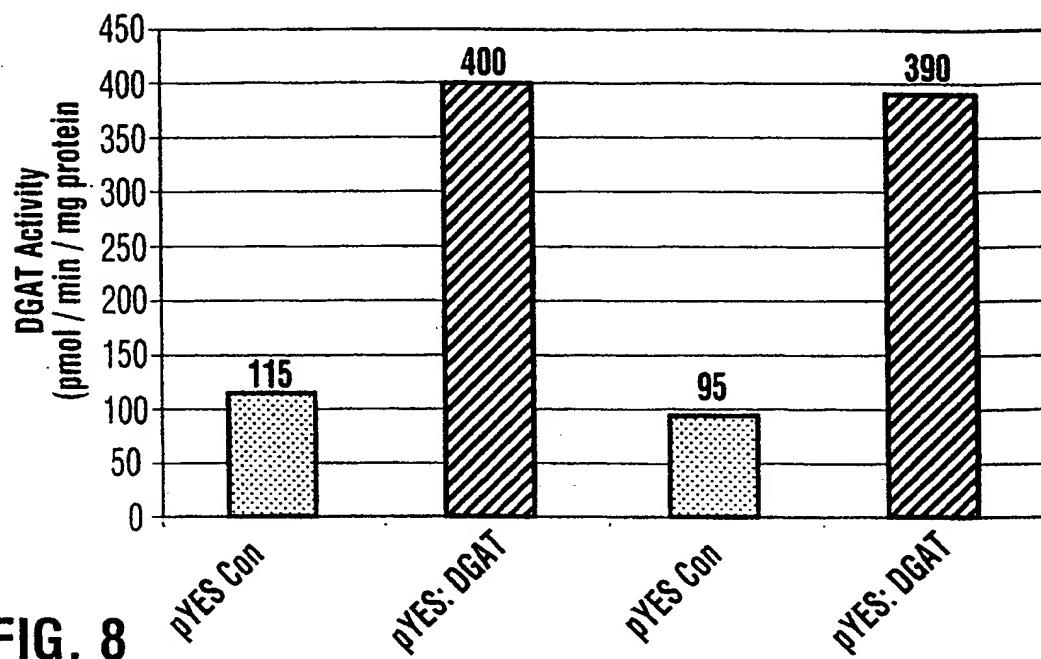


FIG. 8

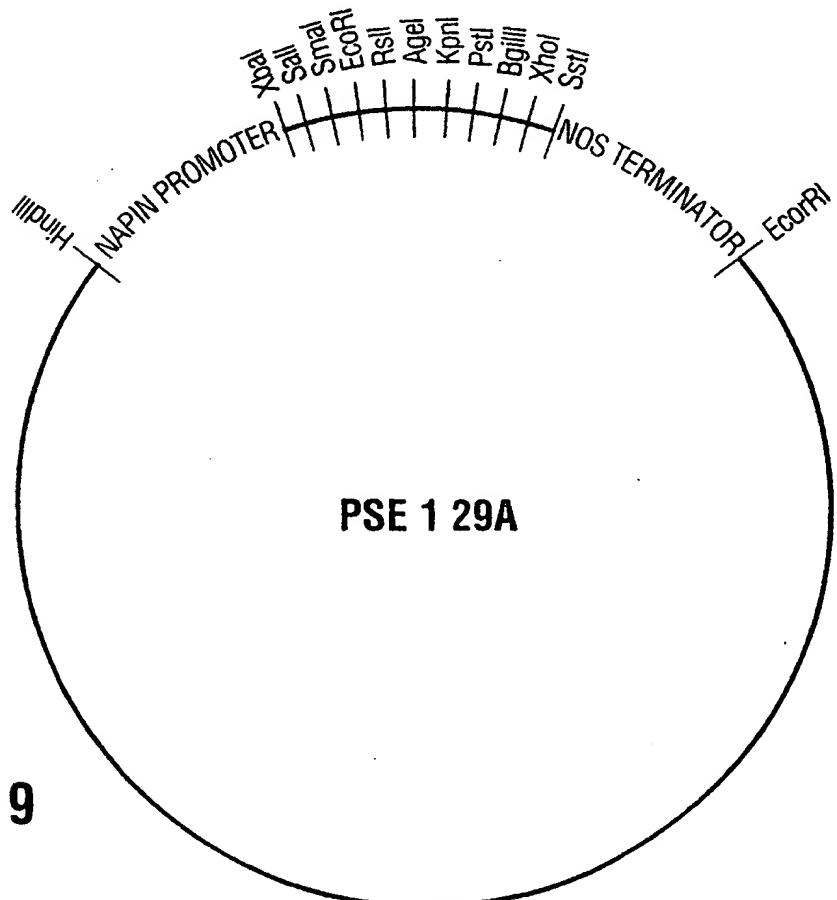


FIG. 9

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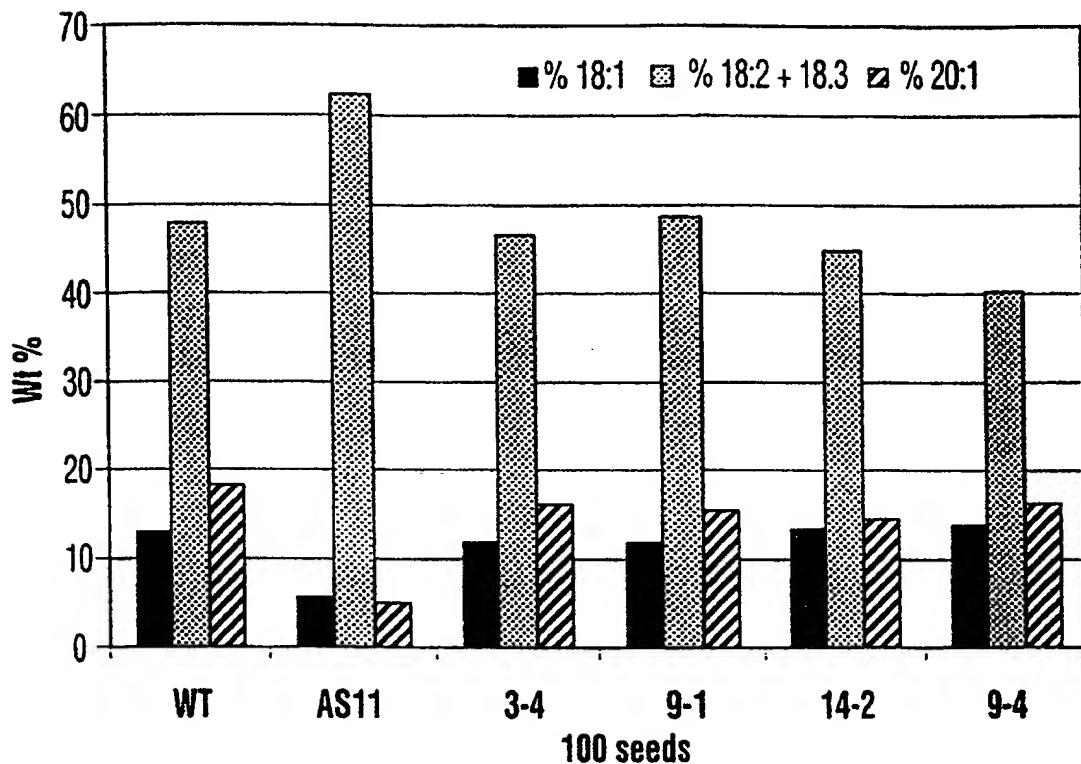


FIG. 10

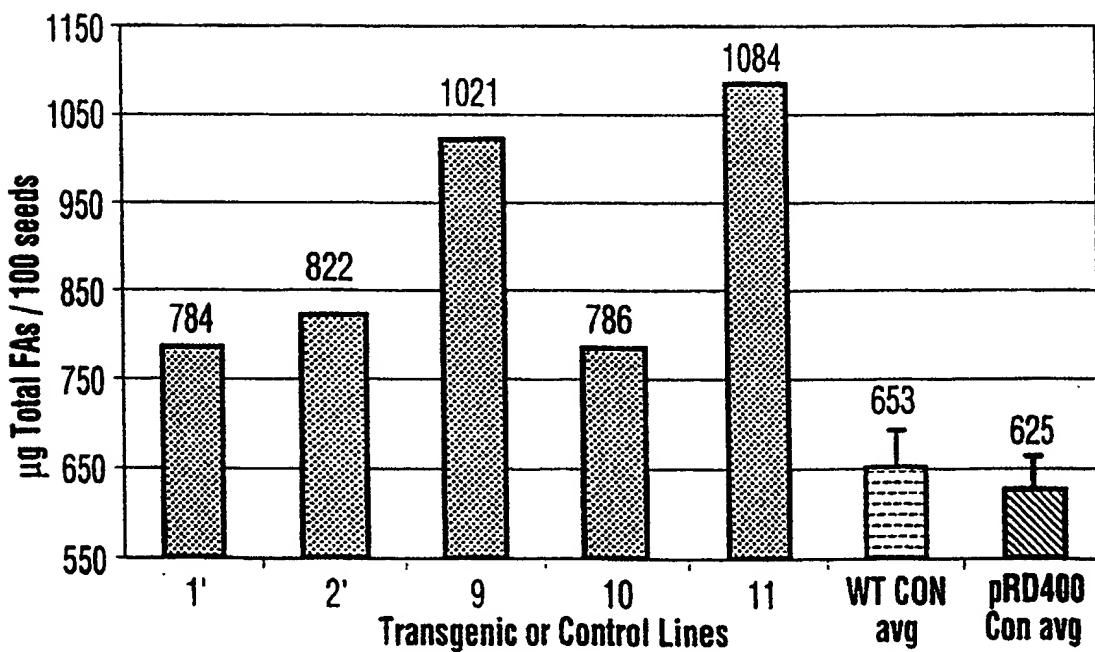


FIG. 11

SUBSTITUTE SHEET (RULE 26)

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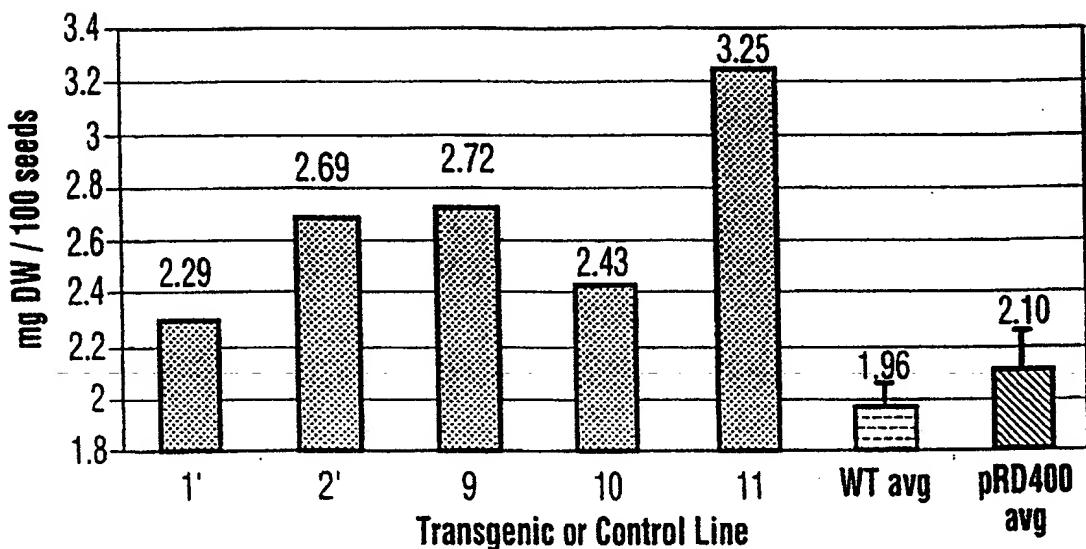


FIG. 12

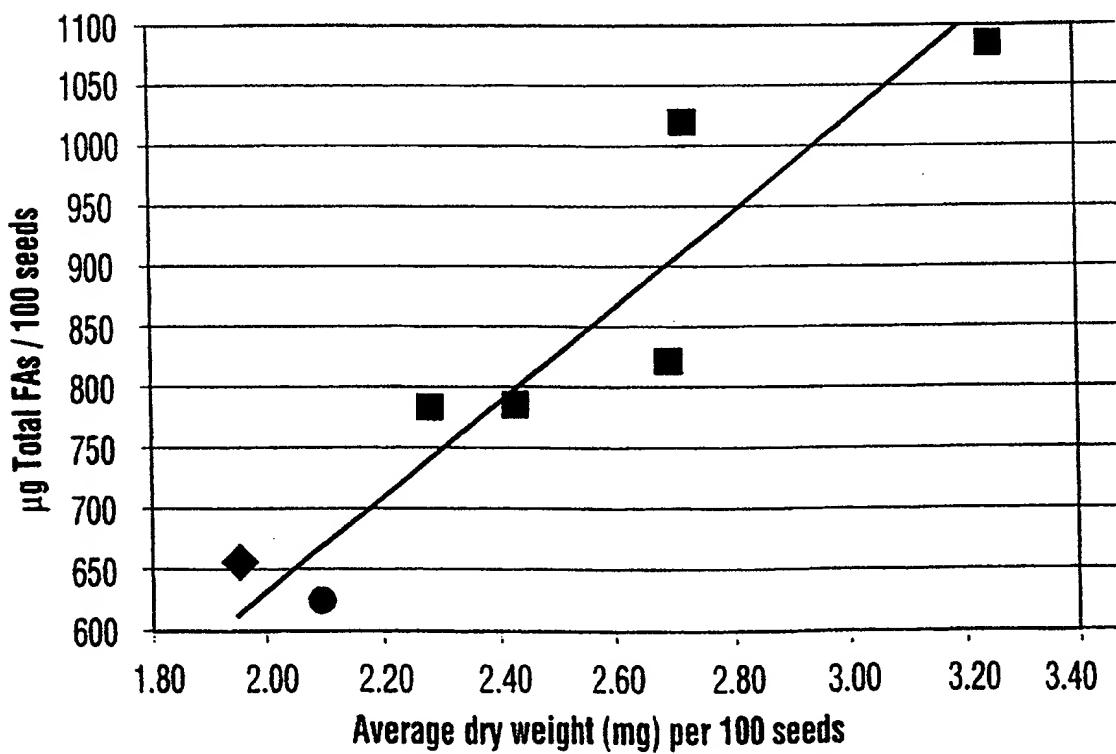


FIG. 13

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## SEQUENCE LISTING

<110> National Research Council of Canada  
Zou, Jitao  
Taylor, David C  
Wei, Yangdou  
Jako, Colette C

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<213> *Arabidopsis thaliana*

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10/20

Glu Lys Leu Val Leu Gln Lys Cys Ile Ser Glu Pro Val Val Ile Phe  
           20                         25                         30

Leu His Val Ile Ile Thr Met Thr Glu Val Leu Tyr Pro Val Tyr Val  
           35                         40                         45

Thr Leu Arg Cys Asp Ser Ala Phe Leu Ser Gly Asp Thr Leu Met Leu  
           50                         55                         60

Leu Thr Cys Ile Val Trp Leu Lys Leu Val Ser Tyr Ala His Thr Asn  
           65                         70                         75                 80

Tyr Asp Ile Arg Thr Leu Ala Asn Ser Ser Asp Lys Ala Asn Pro Glu  
           85                         90                         95

Val Ser Tyr Tyr Val Ser Leu Lys Ser Leu Ala Tyr Phe Met Leu Ala  
           100                       105                         110

Pro Thr Leu Cys Tyr Gln Pro Ser Tyr Pro Arg Ser Pro Cys Ile Arg  
           115                       120                         125

Lys Gly Trp Val Ala Arg Gln Phe Ala Lys Leu Val Ile Phe Thr Gly  
           130                       135                         140

Leu Met Gly Phe Ile Ile Glu Gln Tyr Ile Asn Pro Ile Val Arg Asn  
           145                       150                         155                 160

Ser Lys His Pro Leu Lys Gly Asp Leu Leu Tyr Ala Ile Glu Arg Val  
           165                       170                         175

Leu Lys Leu Ser Val Pro Asn Leu Tyr Val Trp Leu Cys Met Phe Tyr  
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Cys Phe Phe His Leu Trp Leu Asn Ile Leu Ala Glu Leu Leu Cys Phe  
           195                       200                         205

Gly Asp Arg Glu Phe Tyr Lys Asp Trp Trp Asn Ala Lys Ser Val Gly  
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Asp Tyr Trp Arg Met Trp Asn Met Pro Val His Lys Trp Met Val Arg  
           225                       230                         235                 240

His Val Tyr Phe Pro Cys Leu Arg Ile Lys Ile Pro Lys Val Pro Ala  
           245                       250                         255

Ile Ile Ile Ala Phe Leu Val Ser Ala Val Phe His Glu Leu Cys Ile  
           260                       265                         270

Ala Val Pro Cys Arg Leu Phe Asn Leu Trp Ala Phe Met Gly Ile Met  
           275                       280                         285

Phe Gln Val Pro Leu Val Phe Ile Thr Asn Phe Leu Gln Glu Arg Phe  
           290                       295                         300

Gly Ser Met Val Gly Asn Met Ile Phe Gly Ser Ala Ser Cys Ile Phe  
           305                       310                         315                 320

11/20

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Lys Gly Ser Met Ser  
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<213> Brassica napus

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35 40 45

Ala Ala Ala Ala Glu Arg Asp Arg Val Asp Ser Ala Ala Glu Glu Glu  
50 55 60

Ala Gln Gly Thr Ala Asn Leu Ala Gly Gly Asp Ala Glu Thr Arg Glu  
65 70 75 80

Ser Ala Gly Gly Asp Val Arg Phe Thr Tyr Arg Pro Ser Val Pro Ala  
85 90 95

His Arg Arg Thr Arg Glu Ser Pro Leu Ser Ser Asp Ala Ile Phe Lys  
100 105 110

Gln Ser His Ala Gly Leu Phe Asn Leu Cys Val Val Val Leu Val Ala  
115 120 125

Val Asn Ser Arg Leu Ile Ile Glu Asn Leu Met Lys Tyr Gly Trp Leu  
130 135 140

Ile Arg Thr Asp Phe Trp Phe Ser Ser Thr Ser Leu Arg Asp Trp Pro  
145 150 155 160

Leu Phe Met Cys Cys Leu Ser Leu Ser Val Phe Pro Leu Ala Ala Phe  
165 170 175

Thr Val Glu Lys Met Val Leu Gin Lys Phe Ile Ser Glu Pro Val Ala  
180 185 190

Ile Ile Leu His Val Ile Ile Thr Met Thr Glu Val Leu Tyr Pro Val  
195 200 205

Tyr Val Thr Leu Arg Cys Asp Ser Ala Phe Leu Ser Gly Val Thr Leu  
210 215 220

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Met Leu Leu Thr Cys Ile Val Trp Leu Lys Leu Val Ser Tyr Ala His  
225 230 235 240

Thr Ser Tyr Asp Ile Arg Thr Leu Ala Asn Ser Ala Asp Lys Val Asp  
245 250 255

Pro Glu Ile Ser Tyr Tyr Val Ser Leu Lys Ser Leu Ala Tyr Phe Met  
260 265 270

Val Ala Pro Thr Leu Cys Tyr Gln Pro Ser Tyr Pro Arg Ser Pro Cys  
275 280 285

Ile Arg Lys Gly Trp Val Ala Arg Gln Leu Ala Lys Leu Val Ile Phe  
290 295 300

Thr Gly Leu Met Gly Phe Ile Ile Glu Gln Tyr Ile Asn Pro Ile Val  
305 310 315 320

Arg Asn Ser Lys His Pro Leu Lys Gly Asp Leu Leu Tyr Ala Ile Glu  
325 330 335

Arg Val Leu Lys Leu Ser Val Pro Asn Leu Tyr Val Trp Leu Cys Met  
340 345 350

Phe Tyr Cys Phe Phe His Leu Trp Leu Asn Ile Leu Ala Glu Leu Leu  
355 360 365

Cys Phe Gly Asp Arg Glu Phe Tyr Lys Asp Trp Trp Asn Ala Lys Ser  
370 375 380

Val Gly Asp Tyr Trp Arg Met Trp Asn Met Pro Val His Lys Trp Met  
385 390 395 400

Val Arg His Val Tyr Phe Pro Cys Leu Arg Ile Lys Ile Pro Lys Val  
405 410 415

Pro Ala Ile Ile Ile Ala Phe Leu Val Ser Ala Val Phe His Glu Leu  
420 425 430

Cys Ile Ala Val Pro Cys Arg Leu Phe Asn Leu Trp Ala Phe Met Gly  
435 440 445

Ile Met Phe Gln Val Pro Leu Val Phe Ile Thr Asn Phe Leu Gln Glu  
450 455 460

Arg Phe Gly Ser Met Val Gly Asn Met Ile Phe Gly Ser Ala Ser Cys  
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13/20

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24

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14/20

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15/20

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<212> DNA  
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24

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24

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21

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33

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16/20

&lt;220&gt;

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&lt;400&gt; 22

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33

&lt;210&gt; 23

&lt;211&gt; 1985

&lt;212&gt; DNA

&lt;213&gt; Arabidopsis thaliana

&lt;400&gt; 23

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 aacgggtggcg gagagttcgt cgatcttgcg aggcttcgtc gacggaaatc gagatccggat 240  
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&lt;210&gt; 24

&lt;211&gt; 5339

&lt;212&gt; DNA

&lt;213&gt; Arabidopsis thaliana

&lt;400&gt; 24

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17/20

18/20

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&lt;211&gt; 547

&lt;212&gt; PRT

&lt;213&gt; Arabidopsis thaliana

&lt;400&gt; 25

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Ser	Asp	Ser	Ser	Asn	Gly	Leu	Leu	Leu	Ser	Gly	Ser	Asp	Asn	Asn	Ser
35								40				45			

Pro	Ser	Asp	Asp	Val	Gly	Ala	Pro	Ala	Asp	Val	Arg	Asp	Arg	Ile	Asp
50									55			60			

Ser	Val	Val	Asn	Asp	Asp	Ala	Gln	Gly	Thr	Ala	Asn	Leu	Ala	Gly	Asp
65								70			75			80	

Asn	Asn	Gly	Gly	Gly	Asp	Asn	Asn	Gly	Gly	Gly	Arg	Gly	Gly	Gly	Glu
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19/20

85

90

95

Gly Arg Gly Asn Ala Asp Ala Thr Phe Thr Tyr Arg Pro Ser Val Pro  
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Ala His Arg Arg Ala Arg Glu Ser Pro Leu Ser Ser Asp Ala Ile Phe  
 115 120 125

Lys Gln Ser His Ala Gly Leu Phe Asn Leu Cys Val Val Val Leu Ile  
 130 135 140

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 145 150 155 160

Gly Leu Phe Asn Leu Cys Val Val Val Leu Ile Ala Val Asn Ser Arg  
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Leu Ile Ile Glu Asn Leu Met Lys Tyr Gly Trp Leu Ile Arg Thr Asp  
 180 185 190

Phe Trp Phe Ser Ser Arg Ser Leu Arg Asp Trp Pro Leu Phe Met Cys  
 195 200 205

Cys Ile Ser Leu Ser Ile Phe Pro Leu Ala Ala Phe Thr Val Glu Lys  
 210 215 220

Leu Val Leu Gln Lys Tyr Ile Ser Glu Pro Val Val Ile Phe Leu His  
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Ile Ile Ile Thr Met Thr Glu Val Leu Tyr Pro Val Tyr Val Thr Leu  
 245 250 255

Arg Cys Asp Ser Ala Phe Leu Ser Gly Val Thr Leu Met Leu Leu Thr  
 260 265 270

Cys Ile Val Trp Leu Lys Leu Val Ser Tyr Ala His Thr Ser Tyr Asp  
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Ile Arg Ser Leu Ala Asn Ala Ala Asp Lys Ala Asn Pro Glu Val Ser  
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Tyr Tyr Val Ser Leu Lys Ser Leu Ala Tyr Phe Met Val Ala Pro Thr  
 305 310 315 320

Leu Cys Tyr Gln Pro Ser Tyr Pro Arg Ser Ala Cys Ile Arg Lys Gly  
 325 330 335

Trp Val Ala Arg Gln Phe Ala Lys Leu Val Ile Phe Thr Gly Phe Met  
 340 345 350

Gly Phe Ile Ile Glu Gln Tyr Ile Asn Pro Ile Val Arg Asn Ser Lys  
 355 360 365

His Pro Leu Lys Gly Asp Leu Leu Tyr Ala Ile Glu Arg Val Leu Lys  
 370 375 380

20/20

Leu Ser Val Pro Asn Leu Tyr Val Trp Leu Cys Met Phe Tyr Cys Phe  
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Phe His Leu Trp Leu Asn Ile Leu Ala Glu Leu Leu Cys Phe Gly Asp  
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Trp Arg Met Trp Asn Met Pro Val His Lys Trp Met Val Arg His Ile  
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Tyr Phe Pro Cys Leu Arg Ser Lys Ile Pro Lys Thr Leu Ala Ile Ile  
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Ile Ala Phe Leu Val Ser Ala Val Phe His Glu Leu Cys Ile Ala Val  
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Pro Cys Arg Leu Phe Lys Leu Trp Ala Phe Leu Gly Ile Met Phe Gln  
485                   490                   495

Val Pro Leu Val Phe Ile Thr Asn Tyr Leu Gln Glu Arg Phe Gly Ser  
500                   505                   510

Thr Val Gly Asn Met Ile Phe Trp Phe Ile Phe Cys Ile Phe Gly Gln  
515                   520                   525

Pro Met Cys Val Leu Leu Tyr Tyr His Asp Leu Met Asn Arg Lys Gly  
530                   535                   540

Ser Met Ser  
545

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0-1	Form - PCT/RO134 (EASY) Indications Relating to Deposited Microorganism(s) or Other Biological Material (PCT Rule 13bis)	
0-1-1	Prepared using	<b>PCT-EASY Version 2.90 (updated 15.10.1999)</b>
0-2	International Application No.	
0-3	Applicant's or agent's file reference	<b>43922-PT</b>
<hr/>		
1	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
1-1	page	<b>32</b>
1-2	line	<b>19</b>
1-3	Identification of Deposit	
1-3-1	Name of depositary institution	<b>American Type Culture Collection</b>
1-3-2	Address of depositary institution	<b>10801 University Blvd., Manassas, Virginia 20110-2209United States of America</b>
1-3-3	Date of deposit	<b>29 November 1999 (29.11.1999)</b>
1-3-4	Accession Number	<b>ATCC PTA-988</b>
1-4	Additional Indications	<b>NONE</b>
1-5	Designated States for Which Indications are Made	<b>all designated States</b>
1-6	Separate Furnishing of Indications	
	These indications will be submitted to the International Bureau later	<b>NONE</b>
<hr/>		
2	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
2-1	page	<b>32</b>
2-2	line	<b>20</b>
2-3	Identification of Deposit	
2-3-1	Name of depositary institution	<b>American Type Culture Collection</b>
2-3-2	Address of depositary institution	<b>10801 University Blvd., Manassas, Virginia 20110-2209United States of America</b>
2-3-3	Date of deposit	<b>29 November 1999 (29.11.1999)</b>
2-3-4	Accession Number	<b>ATCC PTA-989</b>
2-4	Additional Indications	<b>NONE</b>
2-5	Designated States for Which Indications are Made	<b>all designated States</b>
2-6	Separate Furnishing of Indications	
	These indications will be submitted to the International Bureau later	<b>NONE</b>
<hr/>		
3	The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on:	
3-1	page	<b>32</b>
3-2	line	<b>21</b>

Original (for SUBMISSION) - printed on 16.12.1999 03:08:17 PM

3-3	<b>Identification of Deposit</b>	
3-3-1	Name of depositary institution	
3-3-2	Address of depositary institution	
3-3-3	Date of deposit	
3-3-4	Accession Number	
3-4	<b>Additional Indications</b>	
3-5	<b>Designated States for Which Indications are Made</b>	
3-6	<b>Separate Furnishing of Indications</b>	
	These indications will be submitted to the International Bureau later	

**FOR RECEIVING OFFICE USE ONLY**

0-4	This form was received with the international application: (yes or no)	<i>yes</i>
0-4-1	Authorized officer	<i>Sophie hadean</i>

**FOR INTERNATIONAL BUREAU USE ONLY**

0-5	This form was received by the international Bureau on:	
0-5-1	Authorized officer	

REC'D 15 MAR 2000

WFO PCT

INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL

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A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>32</u> , line <u>21</u>	
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## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/CA 99/01202

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N15/54 C12N15/82 A01H5/00 A01H5/10 A01H3/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	R61u012 Database Entry Ac005917 Accession number AC005917; 4 November 1998 LIN X. ET AL.: "Arabidopsis thaliana chromosome II section 113 of 255 of the complete sequence" XP002133608 nucleotides 21090-23400 -& LIN X. ET AL. : "Sequence and analysis of chromosome II of Arabidopsis thaliana" NATURE, vol. 402, 1999, pages 761-768, XP000877287 LONDON GB ----- -/-	1-4, 6-8, 10-13, 19-23

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
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\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

30 March 2000

Date of mailing of the international search report

14/04/2000

Name and mailing address of the ISA

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Authorized officer

Montero Lopez, B

## INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 99/01202
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CASES S ET AL: "Identification of a gene encoding an acyl CoA: diacylglycerol acyltransferase, a key enzyme in triacylglycerol synthesis"</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE, WASHINGTON, vol. 95, no. 22, 27 October 1998 (1998-10-27), pages 13018-13023, XP002122745</p> <p>ISSN: 0027-8424</p> <p>cited in the application the whole document</p> <p>---</p>	1,2,22
X	<p>VESNA KATAVIC ET AL.: "Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in Arabidopsis thaliana affecting diacylglycerol acyltransferase activity"</p> <p>PLANT PHYSIOLOGY, vol. 108, 1995, pages 399-409, XP002915657</p> <p>cited in the application page 399, right-hand column, last paragraph -page 400, left-hand column, paragraph 1 page 402, left-hand column, paragraph 2 -right-hand column, paragraph 1 page 403, right-hand column, last paragraph -left-hand column, paragraph 1 page 405, right-hand column, last paragraph -page 408, left-hand column, paragraph 1</p> <p>---</p>	10-18
E	<p>WO 99 67403 A (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA)</p> <p>29 December 1999 (1999-12-29)</p> <p>page 3, line 27 -page 6, line 5 page 10, line 2 - line 25 page 14, line 16 - line 21 page 15, line 24 -page 16, line 28 page 33, line 4 - line 32 page 39, line 28 -page 40, line 11</p> <p>---</p>	1-4,6, 10,12, 19-23
E	<p>WO 00 01713 A (CALGENE LLC)</p> <p>13 January 2000 (2000-01-13)</p> <p>page 4, line 10 - line 17 page 4, line 20 -page 5, line 2 page 11, line 27 -page 24, line 21; examples 11-14,16</p> <p>---</p>	1-4,6, 10,12, 19,22,23
		-/-

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/01202

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Empln Database Entry Ath238008 Accession number AJ238008; 18 June 1999 ZOU J. ET AL.: "The Arabidopsis thaliana TAG1 gene encodes for a diacylglycerol acyltransferase" XP002133609 cited in the application the whole document	1,2,22
P,X	ZOU, JITAO ET AL: "The Arabidopsis thaliana TAG1 mutant has a mutation in a diacylglycerol acyltransferase gene" PLANT J. (1999), 19(6), 645-653 , XP002133607 the whole document	1,2,22
P,X	EMBL Database Entry AF155224 Accession number AF155224; 30 June 1999 NYKIFORUK C.L. ET AL.: "Brassica napus putative diacylglycerol cyltransferase (DGAT2) mRNA" XP002133639 cited in the application the whole document & NYKIFORUK C.L. ET AL.: "Isolation and sequence analysis of a novel cDNA encoding a putative diacylglycerol acyltransferase from a microspore-derived cell suspension culture of Brassica napus L. cv Jet Neuf (Accession No. AF155224) (PGR99-123)." PLANT PHYSIOLOGY, vol. 120, no. 4, 1999, pages 1207-1207, LONDON GB	1,2,22
P,X	EMBL Database Entry AF164434 Accession number AF164434; 26 July 1999 NYKIFORUK C.L. ET AL.: "Brassica napus putative diacylglycerol acyltransferase (DGAT1) mRNA" XP002133640 cited in the application the whole document & NYKIFORUK C.L. ET AL.: "Isolation and characterization of a cDNA encoding a second putative diacylglycerol acyltransferase from a microspore-derived cell suspension culture of Brassica napus L. cv Jet Neuf (Accession No. AF164434) (PGR99-158)" PLANT PHYSIOLOGY, vol. 121, no. 3, 1999, pages 1053-1053,	1,2,22
-/--		

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 99/01202

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>HOBBS D H ET AL: "Cloning of a cDNA encoding diacylglycerol acyltransferase from <i>Arabidopsis thaliana</i> and its functional expression"            FEBS LETTERS, NL, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM,            vol. 452, no. 3,            11 June 1999 (1999-06-11), pages 145-149,            XP002122747            ISSN: 0014-5793            the whole document</p>	1,2,22
P,X	<p>WO 99 63096 A (CALGENE LLC)            9 December 1999 (1999-12-09)</p> <p>page 3, line 24 -page 4, line 17            page 6, line 13 -page 8, line 16            page 12, line 30 -page 20, line 10;            examples 1-4,7,8            sequence listing SEQ ID NO:1</p>	1-4,6, 10-13, 19,22,23

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International Application No

PCT/CA 99/01202

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9967403 A	29-12-1999	WO 9967268 A	29-12-1999
WO 0001713 A	13-01-2000	NONE	
WO 9963096 A	09-12-1999	NONE	

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